ISOLATION AND CHARACTERISATION OF MERCURY- AND ANTIBIOTIC-RESISTANT ORAL BACTERIA

Thesis submitted by
Rachel Pike
for the degree of
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in the
Faculty of Biology
University of London

Department of Microbiology
Eastman Dental Institute
for Oral Health Care Sciences
256 Gray's Inn Road
London
WC1X 8LD

-2005-

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

As very little information was available on mercury sensitivity testing, the first aim was to determine the most suitable agar and concentration of mercuric chloride to use in this project. The primary objective of the study was to determine whether mercury released from amalgam fillings could increase the prevalence of mercury-resistant bacteria in the oral flora of children. This was achieved through cross-sectional and longitudinal studies. The second aim was to determine whether changes in mercury resistance correlated with changes in the incidence of antibiotic resistance. The final aim was to determine whether individual mercury-resistant isolates contained the *merA* gene.

In the cross-sectional study, saliva and plaque samples were collected from patients with and without amalgam fillings. No significant differences in the proportion of mercury-resistant bacteria were detected between the two groups. One hundred and thirty nine mercury-resistant bacteria were isolated and 41% (with amalgam) and 33% (without amalgam) of these were also resistant to one or more antibiotics. Resistance to tetracycline was most common.

Sixteen patients were enrolled into the longitudinal study. The proportions of mercury-and antibiotic-resistant bacteria were determined on 3 separate occasions (2 pre-amalgam and 1 post-amalgam). There was not a statistically significant change in the incidence of mercury- or antibiotic-resistant bacteria during the month after the installation of the amalgam fillings. However, a linear association between the number of surfaces and proportion of mercury-resistant bacteria was

observed. Eighty eight mercury-resistant bacteria were isolated and 27% (pre-amalgam) and 40% (post- amalgam) of these were resistant to one or more antibiotics. Resistance to erythromycin was most common.

One hundred and thirty two mercury-resistant bacteria were screened for the *merA* gene using PCR and 2 sets of primers. Sixty three percent of the streptococci were found to contain the *merA* gene. Coagulase-negative staphlyococci, *Rothia dentocariosa* and *Neisseria* species contained the *merA* gene, while *Staphylococcus aureus* and *Pseudomonas stutzeri* did not. All sequenced amplicons were found to be up to 95% identical to the *Bacillus cereus* RC607 *merA* gene.

The results of this study have failed to demonstrate any definitive link between the presence of mercury amalgam in teeth and the presence, or proportion, of mercury- or antibiotic-resistant bacteria in the oral cavity of children.

DECLARATION

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated.

The statistical analysis was performed by Dr Mark Gilthorpe and Dr Aviva Petrie of the Eastman Dental Institute.

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ABBREVIATIONS

ATCC	American Type Culture Collection
BHI	Brain Heart Infusion Broth
BHIA	Brain Heart Infusion Agar
BSAC	British Society for Antimicrobial Chemotherapy
CA	Columbia Agar
CBA	Columbia Blood Agar
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
CH_3Hg^+	Methylmercury
CNS	Coagulase-negative staphylococcus
CO_2	Carbon dioxide
Cys	Cysteine residue
DNA	Deoxyribonucleic Acid
EMSA	Ethylmercurithiosalicylate
ESBL	Extended-spectrum β lactamase
Erm (*erm*)	Erythromycin resistant methylase (*gene*)
FAD	Flavin adenine dinucleotide
GR	Glutathione reductase
Hg^0	Metallic mercury
Hg^{2+}	Divalent mercuric ions
$HgCl_2$	Mercuric chloride
HPA	Health Protection Agency (formerly known as the Public Health Laboratory Service)
H_2S	Hydrogen sulphide
IQR	Interquartile range
ISOA	Iso-Sensitest Agar
log	natural logarithm
LPD	Lipoamide dehydrogenase
Mef (*mef*)	Macrolide efflux protein (*gene*)
MerA (*merA*)	Mercury reductase (*gene*)
MerB (*merB*)	Mercurial lyase (*gene*)
MerC (*merC*)	Transport protein (*gene*) expressed by the *mer* operon

MerD (*merD*)	Regulatory protein (*gene*) expressed by the *mer* operon
MerE (*merE*)	Transport protein (*gene*) expressed by the *mer* operon
MerF (*merF*)	Regulatory protein (*gene*) expressed by the *mer* operon
MerG (*merG*)	Protein (*gene*) involved in broad-spectrum mercury-resistance
MerO	*Mer* operon operator
MerP (*merP*)	Transport protein (*gene*) expressed by the *mer* operon
MerT (*merT*)	Transport protein (*gene*) expressed by the *mer* operon
μg	microgram
μg/ml	microgram per millilitre
mg	milligram
mg/l	milligram per litre
μM	Micromolar
MHA	Mueller-Hinton Agar
MIC	Minimum Inhibitory Concentration
MIC_{50}	The MIC at which 50% of the tested isolates are inhibited
MIC_{90}	The MIC at which 90% of the tested isolates are inhibited
MLS_B	Macrolides, lincosamides and type B streptogramins
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mitis Salivarius Agar
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnological Information
NCTC	National Culture Type Collection
NCCLS	National Committee for Clinical Laboratory Standards
Otr (*otr*)	Oxytetracycline resistance determinant (*gene*)
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
p-HMB	*Para*-hydroxymercurobenzoate
PK	Pharmacokinetics
PMA	Phenylmercuric acetate
PMN	Polymorphonuclear lymphocytes
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid

SD	Standard Deviation
Tet (*tet*)	Tetracycline resistance determinant (*gene*)
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
TR	Thioredoxin
VRE	Vancomycin-resistant enterococcus/enterococci

PUBLICATIONS RESULTING FROM THIS THESIS

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Pike, R., Lucas, V., Stapleton, P., Gilthorpe, M. S., Roberts, G., Rowbury, R., Richards, H., Mullany, P. & M. Wilson, 2002, Prevalence and antibiotic resistance profile of mercury-resistant oral bacteria from children with and without mercury amalgam fillings. *Journal of Antimicrobial Chemotherapy*, **49**, 777-783.

Pike, R., Lucas, V., Petrie, A., Roberts, G., Stapleton, P., Rowbury, R., Richards, H., Mullany, P. & M. Wilson, 2003, Effect of restoration of children's teeth with mercury amalgam on the prevalence of mercury- and antibiotic-resistant oral bacteria. *Microbial Drug Resistance*, **9**, 93-97.

Stapleton, P., Pike, R., Mullany, P., Lucas, V., Roberts, G., Rowbury, R., Wilson, M. & H. Richards, 2004, Mercuric resistance genes in gram-positive oral bacteria. *FEMS Microbiology Letters*, **236**, 213-220.

Stapleton, P., Adams, V., Pike., R., Lucas, V., Roberts, G., Mullany, P., Rowbury, R., Wilson, M. & H. Richards, 2004, Characterisation of viridans group streptococci with different levels of Tet(M)-mediated tetracycline resistance. *International Journal of Antimicrobial Agents*, **24**, 439-443.

Chapter One

Introduction

1.0 Introduction

1.1 The Oral Microflora

The oral cavity is a highly varied environment with diverging environmental determinants. This results in a complex resident microbiota consisting of a wide range of bacterial genera, as well as viruses, yeasts and sometimes protozoa (Schuster, 1999). Although the ecological conditions within the mouth are constantly changing, throughout adulthood the resident oral microflora remains relatively stable (Liljemark & Bloomquist, 1996). However, transient fluctuations in the oral flora often occur during certain circumstances. These include changes in saliva flow rates, variations in the frequency and types of foods eaten, tooth-brushing and antibiotic therapy (Joyston-Bechal *et al*, 1992, Epstein *et al*, 1998). Permanent changes in the mouth, such as the extraction of teeth, the insertion of protheses (dentures) and dental treatment (fillings) can also result in a more permanent change in the resident oral flora (Wilson, 1998, Lockhart *et al*, 1999).

The oral ecosystem encounters most variation during childhood. The oral cavity of the foetus in the womb is normally sterile, but after birth is quickly colonised by organisms deriving from the mother and other individuals in close proximity to the child (Alaluusua, 1991, Li & Caulfield, 1995). During feeding the mouth is regularly inoculated with microorganisms resulting in the establishment of pioneer species (Mohan *et al*, 1998). The neonatal oral cavity offers only epithelial (mucosal)

surfaces for colonisation and the predominant organism found within a newborn's mouth is *Streptococcus salivarius*, an organism which is present on the tongue dorsum in adulthood (McCarthy *et al*, 1965, Smith *et al*, 1993). Once the pioneer species have become established, the oral environment changes due to the metabolic activity of the organisms, resulting in conditions suitable for colonisation by a succession of other organisms. These pioneer populations are generally aerobes and facultative anaerobes, including streptococci, staphylococci, *Neisseria* and *Veillonella* species (Pearce *et al*, 1995, Bloomquist *et al*, 1996). With increasing age there is an increase in non-sporulating anaerobes, filamentous forms and yeasts such as *Actinomyces*, *Lactobacillus*, *Rothia*, *Fusobacterium*, *Bacteroides*, *Leptotrichia*, *Corynebacterium* and *Candida* (McCarthy *et al*, 1965, Hannula *et al*, 1999). Teeth provide a wide range of habitats for microorganisms and at 6-10 months of age the central incisors erupt presenting a habitat for organisms with a high affinity for hard surfaces. After tooth eruption, the environment of the oral cavity becomes similar to that of an adult. Initially, *Streptococcus sanguinis* and mutans streptococci (*S. mutans* and *S. cricetus*) colonise the tooth. The development of teeth also results in the presence of gingival crevicular fluid (GCF), a serum-like exudate, producing a nutrient source for organisms (Berkowitz *et al*, 1975, Morhart & Fitzgerald, 1976, Smith *et al*, 1993). The gingival crevice has a low oxygen concentration and is therefore populated by anaerobic organisms such as spirochaetes and black-pigmented anaerobes (Marsh & Bradshaw, 1997, Tanaka *et al*, 1998). Primary dentition is usually complete by 3 years of age, while permanent teeth begin to develop at the age of 6 years (Bishara *et al*, 1988, Ranly, 1998). Permanent tooth eruption is usually

complete by 19 years of age, although the third molars (wisdom teeth) may not erupt at all (Daito *et al*, 1992). After the secondary teeth have erupted the oral flora generally remains stable. However, hormonal changes that occur during adolescence and pregnancy can result in these hormones entering the gingival crevice and an increase in the number of spirochaetes and black-pigmented anaerobes (Bailit *et al*, 1964).

During adulthood the bacterial populations exist in harmony with the host, remaining relatively stable unless major physiological changes are encountered. Complete loss of teeth results in a reduction or the elimination of spirochaetes and a reduction in lactobacilli, yeasts and *S. sanguinis* while wearing dentures can promote the colonisation of *Candida albicans,* but eliminate *S. sanguinis* (Socransky & Manganiello, 1971, Millsap *et al*, 1999). In addition, oral candidiasis is more common in the elderly due to changes in the oral mucosa, malnutrition and trace element deficiencies, while antibiotic therapy often results in an increase in the presence of yeasts (Hannula *et al*, 1999, Lockhart *et al*, 1999). Dietary habits can also perturb the balance of the oral microflora. A diet high in carbohydrates can result in an increase in aciduric and cariogenic organisms in the mouth such as mutans streptococci and lactobacilli (van Houte, 1994).

1.2 Plaque formation

After tooth eruption, several organic deposits may form on the surface of teeth. These deposits include pellicle, materia alba, plaque and calculus and also form soon after tooth-brushing. Pellicle forms first and occurs when salivary proteins adsorb to the hydroxyapatite (enamel) surface of teeth (Lie, 1978). This results from electrostatic ionic interactions between phosphate groups and calcium ions on the enamel surface and oppositely-charged groups in the salivary macromolecules (Gristina, 1987, Dowd, 1999). The resulting pellicle is heterogenous with a thickness of 100nm at 2 hours and 500-1000nm at 24-48 hours. The pellicle is very tenacious and initially attracts coccal organisms such as streptococci to the tooth surface through hydrophobic interactions and electrostatic forces (Jenkinson & Lamont, 1997). At this point, the pellicle has now become plaque. *Streptococcus sanguinis* is a prominent organism amongst the earliest colonisers of the tooth surface, deriving from saliva (Socransky *et al*, 1977). This organism initially adheres to small irregularities and fissures that are relatively sheltered from oral cleansing forces (saliva). Other pioneer organisms include *Neisseria* species, *Streptococcus oralis* and *Streptococcus mitis* (Figure 1.1). If the plaque accumulates undisturbed, the proportions of organisms present in the biofilm changes. Between 0-7 days, streptococci are the predominant organisms, but by day 14 the oxidation-reduction potential has decreased and anaerobic rods and filamentous organisms predominate (Listgarten *et al*, 1975). Another important mechanism that encourages species diversity and allows plaque build up is coaggregation between organisms

(Hughes *et al*, 1988). Carbohydrate-binding proteins known as lectins are found on bacterial surfaces and interact with complementary carbohydrate-containing receptors on other microorganisms (Herp *et al*, 1988). Intra-generic and inter-generic coaggregation between the pioneer *Streptococcus* species and *Actinomyces* species results in further bacterial accumulation in plaque (Jenkinson, 1994). This results in further inter-generic coaggregation between the primary colonisers and other genera.

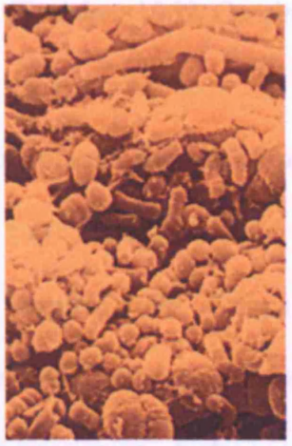

Figure 1.1: A Bacterial Matrix (Plaque)

The organisms in dental plaque are embedded in an organic matrix, which accounts for approximately 30-70% of the total plaque volume (Socransky *et al*, 1963). The organic matrix consists of salivary glycoproteins and extracellular polysaccharides produced by the bacteria found within the biofilm (De Jong & Van der Hoeven, 1987, Bowden & Li, 1997). Organisms such as *S. mutans* produce several types of extracellular polysaccharides, known as dextran (a glucan), from dietary sucrose (Morhart & Fitzgerald, 1976, Ruby *et al*, 1978, Kuramitsu & Wondrack, 1983).

Dextran has been shown to be an important parameter in holding this species together *in vitro* and *in vivo* (Jordan & Keyes, 1966, Gibbons, 1968). Other organisms such as *S. salivarius*, convert dietary sucrose into fructan (levan), which is used as an energy source by other organisms (Ebisu *et al*, 1975, Kelstrup, 1981, Cerning, 1990). Although most work on the origin of plaque matrix carbohydrates has been confined to streptococci, other genera such as *Neisseria*, *Actinomyces*, *Lactobacillus* and *Micrococcus mucilagenosus* are also known to produce extracellular materials that contribute to the plaque matrix (Bowden, 1969, Cerning, 1990).

The organisms found in dental plaque live in close proximity to each other and although often beneficial to each other, can also be antagonistic (Vernazza & Melville, 1979, Shapiro, 1996, Tompkins *et al*, 1997). Antagonistic factors include essential nutrient depletion and the accumulation of inhibitory and toxic by-products, such as bacteriocins, hydrogen peroxide and organic acids. Organic acids reduce the pH and these acidic conditions can lead to the development of caries (Shapiro, 1996, Tompkins *et al*, 1997, Barnard & Stinson, 1999).

Antagonistic factors and nutrient competition leads to environmental heterogeneity, which allows organisms with different growth requirements to grow within the biofilm, and also ensures synergistic co-existence of different species (Sundqvist, 1994). For example, some oral spirochaetes require spermine, spermidine and putrescine, which are provided by Gram-positive rods and fusobacteria (Loesche, 1968, Deyloff & Sanders, 1980). *Veillonella* and oral diphtheroids produce

vitamin K, which is an essential growth requirement for the black-pigmented anaerobes (Mayrand *et al*, 1980, Shah & Collins, 1983).

The quantitative composition of plaque can vary quite markedly at different areas in the mouth and at different sites on the same tooth (Socransky *et al*, 1977). For example, gingival crevice plaque has a low oxidation-reduction potential, resulting in high species diversity, especially of anaerobes, although the total number of bacteria can be low (Mettraux *et al*, 1984). In addition, anatomical factors, such as malalignment, may predispose areas of the mouth to excessive plaque accumulation (Quirynen, 1994). Similarly orthodontic or prosthetic appliances may interfere with oral hygiene procedures and encourage plaque formation (Chadwick, 1994, Heintze *et al*, 1996).

The plaque biofilm can protect organisms within it from saliva, mastication and antimicrobials (Marsh, 1989, Bowden & Li, 1997). The matrix acts as a diffusion-limiting barrier and the sensitivity of a biofilm-associated organism to antibiotics and disinfectants is reduced when compared to the same organism in suspension (Wilson, 1996).

1.3 The Oral Microflora and Caries

1.3.1 Dental Caries

The plaque matrix also plays an important role in caries development since harmful bacterial products such as lactic acid are retained in high concentrations at particular sites where they can initiate caries (McNee *et al*, 1982). Dental caries is the most common disease in man, although its prevalence and incidence is decreasing in developed countries, but increasing in developing countries where consumption of dietary carbohydrates has increased (Newbrun, 1992, Woodward & Walker, 1994, Ismail *et al*, 1997).

Organisms associated with caries formation are mutans streptococci and lactobacilli (Schuster, 1999). These organisms possess the ability to rapidly transport sugars and convert them to acid and they can also tolerate acidic conditions for prolonged periods and are able to continue to metabolise and multiply at low pH (Bender *et al*, 1986, Belli & Marquis, 1991, Miyagi *et al*, 1994, Bradshaw & Marsh, 1998).

Cariogenic organisms are usually found in small numbers in plaque at neutral pH, but when the frequency of fermentable carbohydrate intake increases, the pH decreases and the number of cariogenic organisms increase. The decrease in pH results in enamel demineralisation (loss of hydroxyapatite crystals)(Figure 1.2)

(Featherstone, 1999). In the presence of fluoride, demineralisation can be reversed and lead to remineralisation (Winston & Bhaskar, 1998, Featherstone, 1999). Additionally, depending on the fluoride concentration and pH of the environment, fluoride can exert bactericidal or anti-enzymatic properties. In the dental clinic, topical applications of fluoride (>1% fluoride) have been shown to be toxic to mutans streptococci (Kay & Wilson, 1988, Marquis, 1995). In oral streptococci, lower concentrations of fluoride can inhibit enzymes involved in acid production and in the transport and storage of glucose and its analogues (Hayes, 1994). The topical application of antimicrobial agents such as chlorhexidine and triclosan can disrupt the ecology of the microflora, although the aims of most agents are anti-plaque rather than anti-caries (Johnson, 1993, Marsh, 1993, Bouwsma, 1996, Gaffar *et al*, 1997, Guggenheim *et al*, 1997). Once the enamel has been demineralised and penetrated, bacteria can gain access directly to the dental tubules and infect the surrounding tissue (Michelich *et al*, 1980, Gutierrez *et al*, 1990). If demineralisation continues, the lesion progresses and a cavity will form, which can result in dissolution of the enamel and transport of the calcium and phosphate ions from the enamel into the surrounding environment (Ingram, 1990). If the lesion is not treated, the cavitation spreads into dentine, often destroying the dental pulp (Figures 1.2 & 1.3). This results in pain and the patient will usually seek treatment.

a b c

Figure1.2: Demineralisation and remineralisation

a) Healthy tooth enamel rods before the acids onslaught, b) Enamel rods demineralised, or broken down, by acid, c) Enamel rods remineralised or rebuilt by fluoride and minerals in saliva

a b c d

Figure 1.3: Cavity formation

a) Tooth decay begins on biting surfaces, between teeth and on exposed roots, b) Untreated, the cavity becomes larger, c) Decays spreads beneath the enamel destroying the tooth surface, d) Decay enters the pulp and an abscess may occur

1.3.2 Restoration of the Caries Lesion

As early as the 7th century, the Chinese used a 'silver paste' containing mercury to fill decayed teeth (Lorscheider *et al*, 1995a). Amalgam fillings have now been an accepted part of dental therapeutics for more than 150 years, constituting approximately 75% of all restorative materials used by dentists (Leinfelder, 1991). The reasons for its popularity include ease of manipulation, relatively low cost and long clinical service life, wearing at a rate similar to that of teeth (Leinfelder, 1991). In addition, hypersensitivity to mercury is extremely rare (Burrows, 1986). However, dental amalgam also has some drawbacks; it is not tooth-coloured and does not easily bond to teeth (Figure 1.4)(Ferracane, 1995). The greatest concern is that amalgam contains mercury, which, if not handled properly, can be a safety hazard to the dental staff and patient (Curtis & Brown, 1992, Kostyniak, 1998).

Figure 1.4: Mercury Amalgam Fillings

Amalgam fillings contain a weight composition that is approximately 50% mercury, 35% silver, 13% tin, 2% copper and trace amounts of zinc (Hahn *et al*, 1989). A newly-placed multisurface dental silver filling involving an occlusal (grinding) surface of a molar tooth contains between 750-1000mg mercury (Hahn *et al*, 1989). It was once believed that the long-term danger to the patient from mercury vapour was remote. Several days after placement, the mercury component of dental amalgam becomes inert, exposing the patient to 1-10μg Hg per day (Lyttle & Bowden, 1993a, Lorscheider *et al*, 1995b). However, studies have shown that tooth-brushing, bruxism (tooth grinding) and gum chewing, especially 'nicotine gum', results in a substantial increase in mercury vapour found in the mouth (Vimy & Lorscheider, 1995a, 1995b). Studies have also shown that plaque may increase mercury release, as bacterial metabolism results in the production of acids which corrode amalgam (Moberg, 1988). Furthermore, symptoms such as fatigue, anxiety, irritability, temper outbursts, stress intolerance, indecision, headaches, depression and a metallic taste in the mouth have been linked to amalgam fillings (Siblerud *et al*, 1994). However, taking these concerns with other biological and environmental safety issues, it has been concluded that dental amalgam presents an acceptable risk-to-benefit ratio when used properly (Corbin & Kohn, 1994, Lorscheider, 1995b).

Mercury amalgam is not the only option available to treat dental caries. Amalgam fillings are preferentially used for the restoration of posterior teeth, while composite, ceramics and hybrid glass-ionomer restorative materials can be used for small class V restorations (Tyas, 1995). With increasing toxicity concerns and aesthetic

considerations, the number of white restorations is significantly increasing (Geurtsen, 1998, Hse *et al*, 1999). However, these materials are often expensive and can only be used for small fillings. Furthermore, they are not resistant to mechanical wear, so can only be used in areas where chewing stress is low (Ferracane, 1995). Additionally, they can shrink which can lead to micro-leakage and secondary caries. The success of the filling depends on the technique employed and, for ceramic inlays, healthy tooth substance must be removed to prevent undercut before the filling can be placed (Rykke, 1992, Ferracane, 1995). Another option is gold fillings. However, they are expensive and, although not as toxic as mercury, gold is absorbed by the body (Rykke, 1992).

1.4 Antimicrobial Activity of Mercury and its Compounds

In addition to treating tooth decay, mercury compounds have been widely used for centuries as disinfectants and antiseptics and as an antimicrobial to treat diseases such as syphilis and leprosy (Blancou, 1995). The use of mercury compounds in medicine has now decreased, although a number of organic derivatives of mercury are used as bacteriostatic and fungistatic agents in vaccine and contact lens preservatives (Winder *et al*, 1980, Halsey, 1999). Until recently, mercury salts were used extensively in the preservation of wood, textiles, paints and leather, as diuretics such as Mersalyl, Meralunde, Chlormerodrin, Mercuramide and Mercaptomerin, in teething powders and also as disinfectants in hospitals (Clarkson, 1990, Lorscheider & Vimy, 1991).

Mercury is toxic to both eukaryotic and prokaryotic cells since it has a high affinity to bind to thiol groups in proteins (Berg & Miles, 1978, Delnomdedieu & Allis, 1993). Organomercurials and inorganic mercury also possess the ability to pass through biological membranes. These properties result in the inactivation of enzymes and damage to membranes. Mercuric ions also bind to nucleotides and lipids and are genotoxic.

1.5 Mercury Resistance

1.5.1 Summary of Previous Work

The presence of mercury in the environment, such as the hospital, environmental contamination, and factories (battery manufacture) is believed to lead to the selection of mercury-resistant bacteria (Timoney *et al*, 1978, Porter *et al*, 1982).

The mercury resistance locus was the first of twelve distinct bacterial plasmid-determined metal resistance loci to be described (Summers *et al*, 1993, Liebert *et al*, 1997). The other metals include arsenic, antimony, boron, cadmium, chromium, cobalt, copper, nickel, silver, tellurium and zinc (Silver & Walderhaug, 1992, Silver & Ji, 1994, Silver, 1996, Silver & Phung, 1996, Silver, 2003). Unlike mercury, many of these resistance loci are found predominantly

in organisms isolated from soil and industrial waste rather than in commensal and pathogenic bacteria isolated from humans and primates (Summers *et al*, 1993).

Bacterial mercury resistance has been studied for decades in laboratories across the world in both environmental and clinical organisms (Moore, 1960, Hall, 1970a, Wireman *et al*, 1997). Most projects have studied links between mercury and multiple antibiotic resistances, especially in clinical isolates. Moore carried out one of the first studies in the 1960s and observed that *Staphylococcus aureus* strains were either resistant or sensitive to a discriminating concentration of mercury salts (Moore, 1960). He found that phage-types of staphylococci associated with hospital epidemics were more often mercury-resistant than non-epidemic strains. In this study, Moore found a close correlation between resistance to mercury salts and to antibiotics in general, but to no antibiotic in particular. He found that mercury-resistant strains of the same phage-type had different antibiograms, thus indicating that mercury resistance and specific antibiotic resistance were independent. In later studies, a high correlation between high penicillinase activity, resulting in penicillin resistance, and mercury resistance was observed (Richmond & John, 1964, Dyke *et al*, 1970). These studies also showed co-transduction of both mercury and penicillin resistance to sensitive *Staph. aureus* (Richmond & John, 1964).

Hall found that mercury resistance at University College Hospital London was common in antibiotic-resistant strains of *Staph. aureus*, although the correlation was not as high as that observed by Moore (Hall, 1970b). Hall found that the number of

mercury-resistant *Staph. aureus* isolated from different hospital wards varied depending on the ward type. For example, of the *Staph. aureus* isolated from nose and wound swabs taken from patients in casualty and outpatient departments only 9% and 20% of the organisms were resistant to mercury. However, 31% and 33% of the *Staph. aureus* isolated from the surgical and medical wards were mercury-resistant. Mercury resistance was found to be common in patients who had been staying long-term in the hospital and rare in patients newly admitted or staying short-term in the hospital.

Since Moore's work 4 decades ago, mercury resistance has been studied in both Gram-negative and Gram-positive species, aerobic and anaerobic organisms, and has been detected in many different genera, such as *Streptococcus*, *Staphylococcus*, *Clostridium*, *Actinomyces*, *Pseudomonas*, *Actinobacillus*, *Salmonella* and *Bacteroides* (Nakahara *et al*, 1977a, Porter *et al*, 1982, Khor & Jegathesan, 1983, Rudrik *et al*, 1985, Avila-Campos *et al*, 1989, Lyttle & Bowden, 1993a).

Rudrik studied obligately anaerobic isolates from both clinical specimens and sewage sludge. In the study, 200 clostridia isolated from the faeces of hospitalised patients and 90 organisms (mainly Enterobacteriaceae) from sewage sludge were screened for mercury resistance (Rudrik *et al*, 1985). The minimum inhibitory concentrations (MIC) of mercuric chloride ($HgCl_2$), phenylmercuric acetate (PMA) and ethylmercurithiosalicylate (EMSA) for 23 *Clostridium perfringens* strains, 5 members of the Enterobacteriaceae family and 2 *Bacteroides ruminicola* ssp *brevis* were

determined using agar dilution. The MICs of the organomercurial compounds, EMSA and PMA, were generally lower than the MICs obtained from the inorganic mercury compound, $HgCl_2$, suggesting the organisms were resistant to inorganic mercuric compounds only. The MICs determined that 3 *C. perfringens* isolates, one *B. ruminicola* ssp *brevis* isolate and all 5 enterobacteriaceae isolates were resistant to $HgCl_2$ although none of these isolates appeared to be resistant to either organomercurial. In this study, none of the resistant anaerobes were shown to contain plasmids, while the resistant facultative isolates (Enterobacteriaceae) contained several plasmids. However, although the data suggested chromosomally-determined mercury resistance, the presence of large plamids or a transposon could not be ruled out. The aerobic organisms showed inducible mercury resistance while the anaerobic organisms did not show inducible mercury resistance. Additionally, the anaerobic organisms did not demonstrate multiple antibiotic resistance, although the Enterobacteriaceae showed resistance to kanamycin, ampicillin, tetracycline and cephalothin.

Avila-Campos and co-authors have worked on mercury resistance in various microbial genera (Avila-Campos *et al*, 1989, Avila-Campos *et al*, 1991a, 1991b). In 1989 the group published a paper in which 41 *Actinobacillus actinomycetemcomitans* strains resistant to mercuric chloride were isolated (Avila-Campos *et al*, 1989). *Actinobacillus actinomycetemcomitans* is an indigenous micro-organism of the human oral cavity but is often associated with periodontal disease, especially in children and young adults. The level of resistance

to mercuric chloride was determined by agar dilution, allowing the determination of the MIC, which was found to be 4μg/ml for all 41 isolates. However, the group stated that this MIC value may not be indicative of a mercury resistance gene, but this organism may have intrinsic resistance. In 1991, the group published another paper in which the stability of the mercuric chloride resistance of *Bacteroides fragilis* strains isolated from human and marmoset (*Callothrix penicillata*) faeces were tested (Avila-Campos *et al*, 1991a). In previous studies, *B. fragilis* has been shown to be resistant to several antibiotics and heavy metals which can be transferred to other intestinal bacteria including Gram-negative facultative anaerobes such as the Enterobacteriaceae (Wallace *et al*, 1981). Avila-Campos and co-authors determined the MIC of 5 *Bacteroides* species by the agar dilution method. All 5 strains tested had an MIC value greater than 2μg/ml, the breakpoint value for mercuric chloride as determined by the authors, suggesting that the strains were all resistant to mercuric chloride. The 5 strains were subcultured for 24 hours in the absence of selective pressure (eg mercuric chloride) and the MIC determined. This procedure was repeated up to 10 times and it was found that after 10 subcultures the MICs remained the same, suggesting that mercury resistance is stable in *Bacteroides* species. This agrees with previous work by Rudrik's group which suggested that mercury resistance in *Bacteroides* species was not plasmid-mediated, while Riley and Mee found that heavy metal resistance in *Bacteroides* was intrinsic rather than plasmid-mediated (Riley and Mee, 1982, Rudrik *et al*, 1985).

Lyttle and Bowden tested selected strains of oral streptococci and *Actinomyces* for their ability to grow in the presence of mercury (Lyttle & Bowden, 1993b). They found that streptococci were more resistant to mercury than *Actinomyces* and that the resistant streptococci adapted to growth in concentrations of mercury greater than on initial isolation. Previous work by these authors showed that oral microbes may play an active role in mercury release from dental amalgam (Lyttle & Bowden, 1993a). They found that biofilms of *S. mutans* facilitated the liberation of mercury from freshly prepared amalgam *in vitro*.

1.5.2 Mercury Resistance Mechanisms

Various mechanisms leading to mercury resistance have been described. Anaerobic organisms such as *Clostridium cochleareum* have the ability to convert mercuric chloride to insoluble mercuric sulphide (HgS) by reaction with hydrogen sulphide (H_2S) (Pan-Hou & Imura, 1981). Detoxification of mercury has also been seen in aerobic mercury-resistant strains of *Bacillus firmus*, *Bacillus pumilus*, *Klebsiella aerogenes* NCTC 418 and *Klebsiella pneumoniae* M426 (Belliveau *et al*, 1991, Essa *et al*, 2002). Certain anaerobic organisms possess the ability to methylate the mercuric ion (Hg^{2+}) to methylmercury (CH_3Hg^+). Methylmercury is volatile and has a very low vapour pressure leading to the compound dissipating from the microbial environment. However, this mechanism is peculiar since methylmercury is more toxic than inorganic mercury salts (mercuric ions)(Hobman & Brown, 1997). Another study has shown that an

Enterobacter aerogenes strain displays a reduced uptake of Hg^{2+} ions (Hobman & Brown, 1997, Essa *et al*, 2002).

However, the most widely reported and studied mechanism of mercury resistance in bacteria is the enzymatic reduction of divalent mercuric ions (Hg^{2+}) to the metallic form (Hg^0). This reaction is catalysed by the cytoplasmic flavoenzyme mercuric reductase (MerA)(Hart *et al*, 1998). The mercuric reductase gene is incorporated in a multigene operon; where the additional genes are involved in regulation of the system (*merR* and *merD*) and in transport of toxic Hg^{2+} to the reductase (*merT*, *merC*, *merE* and *merF*). At present, the best-understood metal resistance loci is that conferring resistance to mercury compounds (Silver & Walderhaug, 1992). Generally, the mercury operon is plasmid encoded, although there are a few exceptions in which the genes are found on the chromosome, such as the *Bacillus* broad-spectrum and *Thiobacillus ferrooxidans* narrow-spectrum resistance operon (Silver & Walderhaug, 1992). Narrow-spectrum resistance is seen in organisms that only have the ability to reduce inorganic mercuric compounds (Hg^{2+}) to Hg^0 and these organisms have a limited range of resistance to organomercurials (Summers, 1986). Some organisms possess broad-spectrum resistance and are able to biotransform both inorganic mercury and a wide range of organomercurials, such as phenylmercuric acetate, methylmercury and *para*-hydroxymercurobenzoate (*p*-HMB) (Summers, 1986, Hobman & Brown, 1997). These organisms possess an organomercurial lyase enzyme which catalyses the cleavage of the organic bond, C-Hg, to yield Hg^{2+} and an organic moiety. Mercuric reductase then reduces Hg^{2+} to

metallic mercury. Broad-spectrum resistance is rarely encountered in Gram-negative organisms (Summers, 1986).

1.5.3 Organisation of Mercury Resistance Genes

The *mer* operon of Gram-negative bacteria have been studied more extensively than those from Gram-positive bacteria, but both possess genes required for regulation, transport and mercury reduction (mercury reductase)(Figure 1.5) (Silver & Phung, 1996, Osborn *et al*, 1997). The *merB* gene (broad-spectrum resistance) is more common in Gram-positive bacteria than Gram-negative bacteria.

The *mer* operon is often a component of transposons and integrons (Misra *et al*, 1984, Kholodii *et al*, 1993, Liebert *et al*, 1999). The first *mer* operon to be studied was found on Tn21, which is present within the multidrug resistant conjugative plasmid NR1 (R100) of *Shigella flexneri*. Tn21 also contains the class I integron In2, which encodes resistance to sulphonamides (*sul*) and streptomycin/spectinomycin (*aadA*). The presence of the integron enables the organism to acquire further antibiotic resistance genes (Hobman *et al*, 2003)(Chapter 1.9.1).

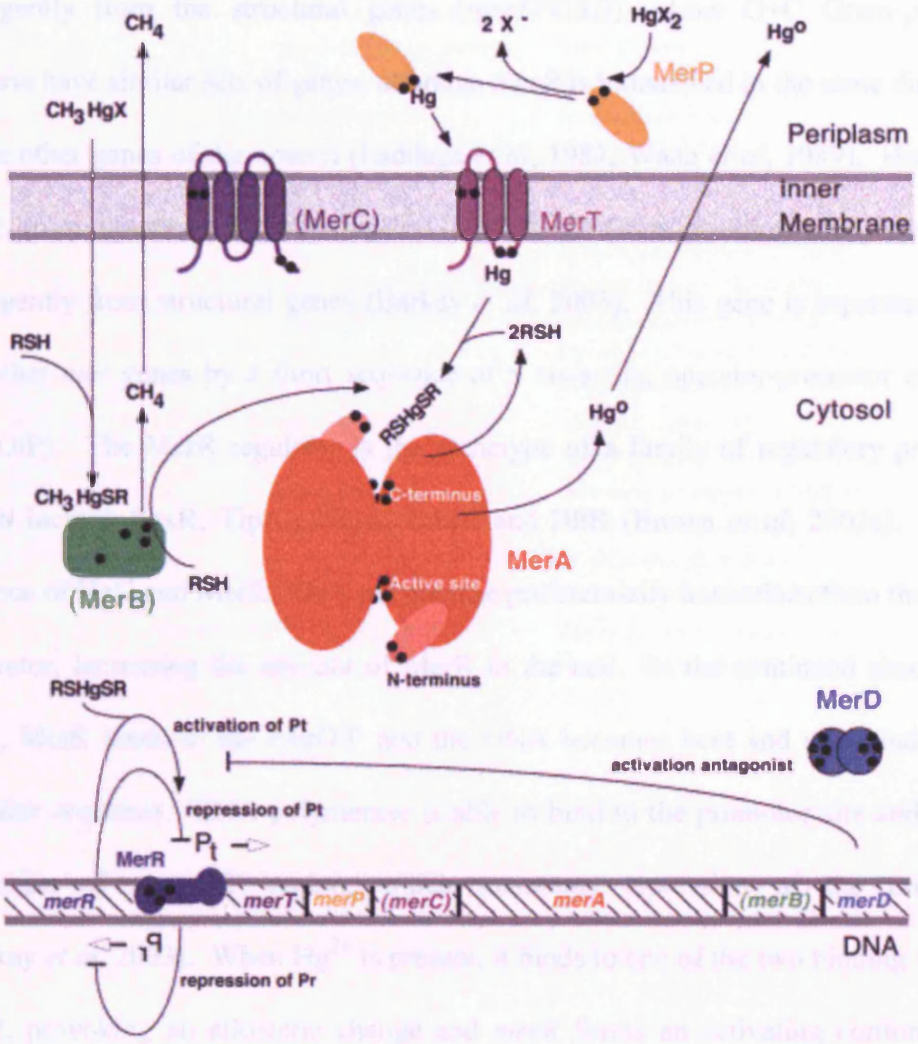

Figure 1.5: Model of a typical Gram-negative *mer* operon and mercury reduction.

The symbol • indicates a cysteine residue (Barkay *et al*, 2003)

The first gene found within the mercury operon is *merR*, which encodes a trans-acting repressor/activator protein, which regulates the expression of the operon (Figures 1.5 & 1.6)(Zeng *et al*, 1998). In Gram-negative organisms, with the exception of the marine bacterium *Pseudomonas haloplanktis*, *merR* is transcribed

divergently from the structural genes (*merTPCAD*). Low G+C Gram-positive bacteria have similar sets of genes, although *merR* is transcribed in the same direction as the other genes of the operon (Laddaga *et al*, 1987, Wang *et al*, 1989). However, *merR* from operons of *Streptomyces* (high G+C Gram-positive) are transcribed divergently from structural genes (Barkay *et al*, 2003). This gene is separated from the other *mer* genes by a short sequence of a cis-acting operator-promotor element (merO/P). The MerR regulator is the archetype of a family of regulatory proteins, which include SoxR, $TipA_L$, NolA, BmrR and BltR (Brown *et al*, 2003a). In the absence of Hg^{2+} and MerR, RNA polymerase preferentially transcribes from the merR promotor, increasing the amount of MerR in the cell. In the continued absence of Hg^{2+}, MerR binds to the merO/P and the DNA becomes bent and unwound at the operator sequence. RNA polymerase is able to bind to the promotor site and forms distortion of the O/P region, which maintains repression of the promotor (Barkay *et al*, 2003). When Hg^{2+} is present, it binds to one of the two binding sites of *merR*, provoking an allosteric change and *merR* forms an activating conformation (Zeng *et al*, 1998). MerR binds tightly to the operator, causing DNA distortion at the centre of the operator and straightens the helix backbone. This allows access of the RNA polymerase to the –35 and –10 transcriptional start site (Brown *et al*, 2003a).

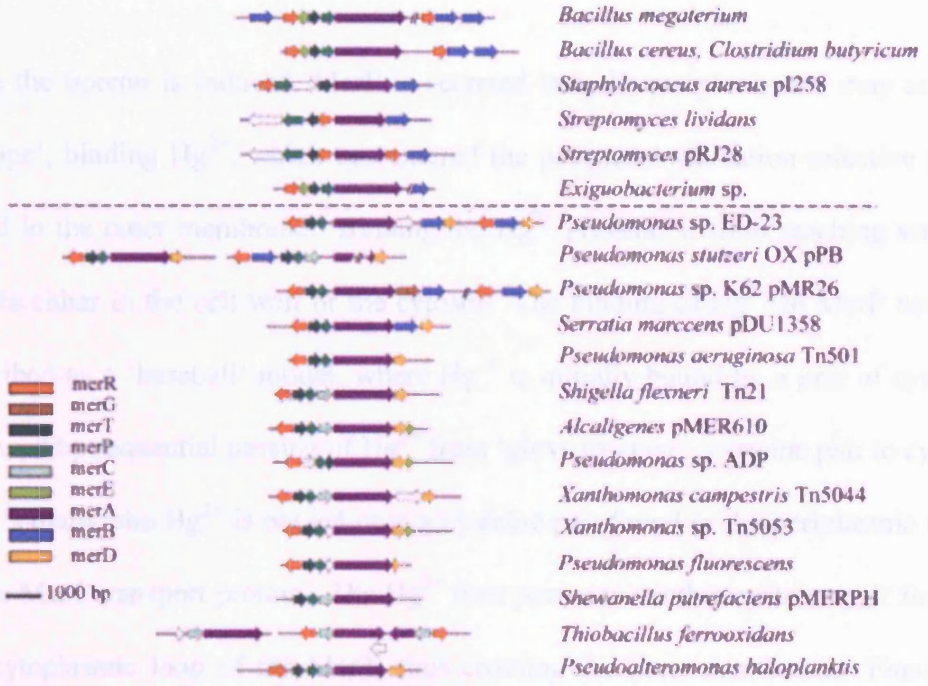

Figure 1.6: Mercury operons from Gram-negative (below line) and Gram-positive (above line) bacteria. Arrows indicate the direction of translation of each gene product (Barkay *et al*, 2003)

A second regulatory protein, MerD, is found downstream from the promotor on the mercury resistance operon. The sequence similarity between MerR and MerD at the NH_2-terminus, which spans the putative DNA-binding domain, suggests that MerD can also bind to an operator site (Mukhopadhyay *et al*, 1991). The group showed that insertion mutations and deletion of the *merD* gene led to greater mercury sensitivity for the host cell (Mukhopadhyay *et al*, 1991). Expression of *merD* is dependent on transcription of the *mer* operon, suggesting that the second regulator only down-regulates expression of the operon (Mukhopadhyay *et al*, 1991).

Once the operon is induced, MerP is secreted into the periplasm and may act as a 'sponge', binding Hg^{2+}, which has entered the periplasm via cation-selective porins found in the outer membrane. Binding the Hg^{2+} prevents it from reaching sensitive targets either in the cell wall or the cytosol. The binding of Hg^{2+} to MerP has been described as a 'baseball' model, where Hg^{2+} is initially bound by a pair of cysteines followed by sequential passing of Hg^{2+} from 'glove to glove', cysteine pair to cysteine pair. Finally, the Hg^{2+} is passed onto a cysteine pair found in the periplasmic region of the MerT transport protein. The Hg^{2+} then passes to another cysteine pair found in the cytoplasmic loop of the MerT, thus crossing the inner membrane. Finally the mercury ion is passed to a cysteine pair in the aminoterminal domain of the mercuric reductase, which is found in the cytoplasm of the bacterial cell (Hamlett *et al*, 1992). The mercuric reductase protein is very specific for mercuric and mercurous ions, and no other metal ion is known to be oxidised or reduced significantly by the enzyme (Misra, 1992). Mercuric reductase belongs to the pyridine nucleotide-disulphide oxidoreductase protein family, of which glutathione reductase (GR), lipoamide dehydrogenase (LPD) and thioredoxin (TR) are members (Hobman & Brown, 1997, Brown *et al*, 2003a). MerA functions as an α_2 homodimer and two pairs of cysteine residues, a redox-active pair (Cys207 and Cys212) from one subunit of MerA and a carboxyl-terminal pair (Cys628 and Cys629) from the other unit of MerA, form the catalytic site (Distefano *et al*, 1990, Liebert *et al*, 1999). MerA uses two hydrides from two NADPH molecules for the

reduction of FAD, which in turn reduces Hg^{2+} bound to the redox-active cysteines of the enzyme (Equation 1.1)(Figure 1.7)(Schiering *et al*, 1991).

$$NADPH + Hg^{2+} = NADP^{+} + H^{+} + Hg^{0}$$

Equation 1.1: Reduction of Hg^{2+} via the reduction of FAD

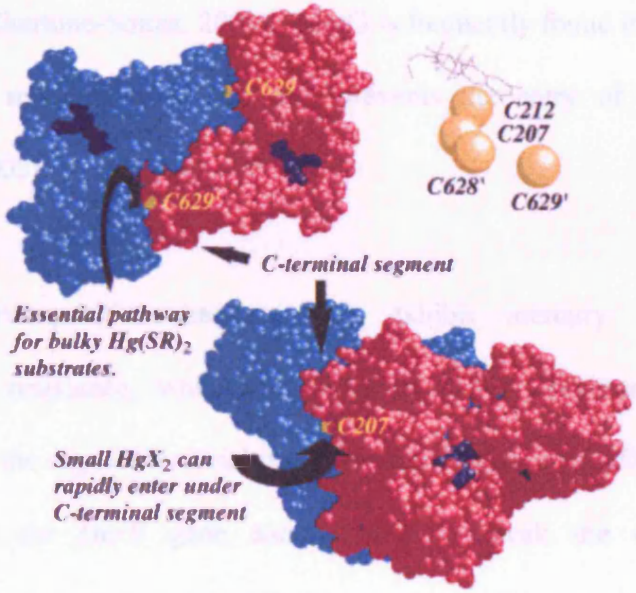

Figure 1.7: Dimeric structure of MerA (Barkay *et al*, 2003)

Some environmental organisms possess the mercuric reductase gene but are mercury-sensitive, suggesting that some of the genes for mercury resistance may be more widespread than the numbers of resistant organisms isolated by mercury

selection show (Bogdanova *et al*, 1992). These organisms may not possess the transport genes required for mercury reduction (Bogdanova *et al*, 1992).

While the function of the MerR, MerT, MerP and MerA have been well-studied, MerC, MerE, MerF and MerG have received less attention (Brown *et al*, 2003b). These genes are not found in all *mer* operons. MerC and MerF are both transport proteins (Liebert *et al*, 1999, Brown *et al*, 2003b, Nascimento & Chartone-Souza, 2003). Very little is known about MerE, but it is thought to be located in the inner membrane of some broad-spectrum mercury-resistant organisms (Barkay *et al*, 2003, Nascimento & Chartone-Souza, 2003). MerG is frequently found in the periplasm of broad-spectrum resistant organisms and prevents the entry of organomercurials (Barkay *et al*, 2003).

Nearly all Gram-positive bacteria that exhibit mercury resistance show broad-spectrum resistance, while only 10% of mercury-resistant Gram-negative bacteria possess the organomercurial lyase enzyme (Barkay *et al*, 2003). This enzyme is encoded by the *merB* gene and is able to break the organic bond in organomercurials (Schottel *et al*, 1974). Organomercurial lyase is a small monomeric protein (22kDa) and has broad substrate specificity for primary, secondary and tertiary alkyl mercuric halides, and for alkyl, vinyl and aryl mercuric halides (Begley *et al*, 1986). Both *in vivo* and *in vitro* organomercurial lyase has a low turnover relative to mercuric reductase, thus preventing the accumulation of toxic inorganic Hg^{2+} in the cell. The enzyme is found in the cytoplasm and binds to a thiol

compound of the organomercurial (Begley *et al*, 1986). The organomercurial lyase forms a complex with the thiol (RHgSR') and this organomercurial-thiolate complex binds to an active cysteine thiolate site found on the enzyme. This leads to the breakage of the C-Hg bond by a proton found in MerB, forming a C-H bond and the organic (R) group is released. The free Hg^{2+} bonds covalently with another cysteine residue until it is released by excess soluble thiol and making it available to mercuric reductase (Barkay *et al*, 2003).

The amino acid sequences of proteins found on the mercury operon (regulation, transport and reduction) have been compared between Gram-positive and Gram-negative organisms. There seems to be high evolutionary conservation suggesting that mercury resistance is an ancient genotype. However, although resistance to mercury compounds was first found on plasmids in Gram-positive bacteria, the phenomenon has been less extensively studied in this class than in the Gram-negative bacteria (Dyke & Richmond, 1967).

1.6 Link between Mercury and Antibiotic Resistance

The possibility of bacteria developing antibiotic resistance was first suggested by Alexander Fleming seven decades ago in his published description of the discovery of penicillin (Fleming, 1929). In most studies, it has been found that over time the number of antibiotic-resistant bacteria has increased and older strains are likely to be less resistant than recent strains. A study involving bacteria isolated from the

'pre-antibiotic' era found that a high percentage of these organisms carried conjugative plasmids that allowed the transfer of DNA from one bacterium to another. In this study, Hughes and Datta investigated the Murray collection, a collection of 433 bacterial strains isolated at the beginning of the 20th century before antibiotic therapy was available (Hughes and Datta, 1983). They found little resistance to antibiotics and to other antimicrobial agents. Only 3 organisms were resistant to mercuric chloride and all 3 were found to possess a plasmid that contained the *mer* operon (Essa *et al*, 2003). In this study the authors concluded that conjugative plasmids were as common in Enterobacteriaceae before the medical use of antibiotics as they are in antibiotic-sensitive strains from the present day. Before this study it was believed that the use of antibiotics encouraged the spread of plasmids (Hughes & Datta, 1983).

For many decades, researchers have studied links between antibiotic and mercury resistance. Richmond and John discovered that genes encoding mercury resistance were plasmid-mediated (Richmond & John, 1964). They isolated a *Staph. aureus* strain from a clinical setting that was resistant to penicillin and mercury salts and a chance observation showed that the loss of penicillinase synthesis was accompanied by the loss of resistance to mercury salts. The correlated loss of penillinase and mercury resistance suggested that the two characters might be closely linked genetically. Using transduction experiments, they concluded that the genes controlling penicillinase synthesis and mercury resistance were closely linked. Relating to previous work by Novick, where penicillinase genes were

plasmid-encoded, Richmond and John concluded that the penicillinase and mercury resistance were on the same plasmid (Novick, 1963, Richmond & John, 1964).

The presence of mercury in the environment has led authors to believe that this can lead to selection pressure, resulting in the emergence and persistence of mercury-resistant organisms. Porter found that the frequency of Hg^{2+} resistance was only 2% for *Staph. aureus* and 9% for *Escherichia coli* in a US hospital (Porter *et al*, 1982). However, in a Japanese hospital, where the use of organomercurials in hospital liquid detergents and disinfectants was heavy, 36% *Staph. aureus* and 57% *E. coli* strains were mercury-resistant. The number of mercury-resistant strains decreased in Japan during later years when the hospital ceased using organomercurials for disinfection purposes. The figures reduced to 10% mercury-resistant *Staph. aureus* and 29% mercury-resistant *Escherichia coli*. During this study the antibiotic susceptibilities of mercury-resistant strains were determined. However, unlike other studies discussed, the results showed no specific antibiotic resistance patterns suggesting that there was no linkage between antibiotic and mercury resistance.

Another Japanese study tested clinical isolates for resistance to heavy metals (mercury, cadmium, arsenic and lead) and antibiotics (streptomycin, tetracycline, chloramphenicol, kanamycin, gentamicin, penicillin, erythromycin and josamycin)(Nakahara *et al*, 1977a). They found that the frequency of heavy metal resistance was the same as, or higher than the frequency of drug resistance. Many

strains were resistant to heavy metals, but remained sensitive to the antibiotics. In this study the authors demonstrated conjugation between a mercury-resistant donor strain and mercury-sensitive recipient strain. Another paper by these authors describes *Pseudomonas aeruginosa* strains expressing multiple heavy metal resistance, including mercury resistance, and multiple antibiotic resistance (Nakahara *et al*, 1977b). However, many isolates were metal-resistant but antibiotic-sensitive.

The work of Groves and colleagues challenges the theory about selection pressure leading to mercury resistance (Groves *et al*, 1975). Groves studied mercury resistance in organisms isolated from Iraqi farmers and families who lived in an area of severe methylmercury poisoning due to the consumption of home-made bread prepared from seed grain treated with a methylmercurial fungicide. Staphylococci were isolated from the anterior nares of 'poisoned' and control populations, which were then tested for resistance to mercury, copper and cadmium. In this study staphylococci isolated from patients exposed to antibiotics were also tested. The authors found that the exposure to methylmercury did not influence the incidence of mannitol-positive (*Staph. aureus*) mercury-resistant strains from the 'poisoned' population, whereas exposure to antibiotics significantly increased the incidence of strains resistant to the mercuric ions. However, in mannitol-negative staphylococci (coagulase-negative staphylococci, CNS) the highest incidence of mercury resistance in staphylococci occurred in the non-exposed population. Resistance to copper ions was more

frequently associated with the presence of *Staph. aureus* whereas the resistance to mercuric ions was most frequently found in coagaulase-negative staphylococci.

1.7 Dental Amalgam and Mercury and Antibiotic Resistance in Oral Bacteria

Several groups have investigated whether the dental amalgam used to repair caries can provide enough selective pressure to promote the emergence and spread of mercury and antimicrobial resistance in the normal human flora (Summers *et al*, 1993, Österblad *et al*, 1995, Edlund *et al*, 1996). Edlund and coauthors studied the resistance patterns in the oral and intestinal flora from patients that had been exposed to mercury due to removal of dental amalgam (Edlund *et al*, 1996). The fillings were replaced with composites, cast gold crowns and inlays. The results were compared against microbes isolated from a control group without any history of amalgam fillings. Edlund found no differences in the resistance pattern of the oral flora between the two groups. However, in the amalgam group there was an increase in the relative number of intestinal organisms resistant to mercury, ampicillin, cefoxitin, erythromycin and clindamycin, although this was calculated to be not statistically significant. In spite of this, a significant correlation between the prevalence of mercury resistance and multiple antibiotic resistance in intestinal bacterial strains such as *E. coli*, *Bacteroides* species and enterococci was observed. In conclusion, the study showed that mercury exposure from amalgam fillings did not seem to be a

major factor in the selection of mercury and antimicrobial resistance in the human oral and intestinal flora.

The results of a study by Österblad and colleagues support the theory that amalgam fillings do not provide selective pressure (Österblad *et al*, 1995). Three patient groups were examined. Group one had never been exposed to dental amalgam fillings, a second group had all their amalgam fillings removed, while the third group had various numbers of amalgam fillings. Aerobic Gram-negative bacilli were isolated from faecal samples and tested for resistance to ampicillin, cefuroxime, nalidixic acid, trimethoprim, suphamethoxazole, tetracycline and mercury. It was found that the patients with a high concentration of Hg^{2+} in their faeces did not have a higher incidence of mercury- or antibiotic-resistant bacteria. However, multiply antibiotic-resistant strains were commonly resistant to mercury. Bacterial conjugation experiments were also carried out, resulting in transfer of mercury and antibiotic resistance from the donor strain to the recipient. All resistance factors found in the mercury-resistant strains, except cefuroxime and nalidixic acid resistance, were transferred and sulphamethoxazole together with tetracycline was the most frequently transferred resistance. No significant difference in transfer frequency between the three subject groups was observed.

In contrast, Summers observed that mercury amalgam provokes an increase in mercury- and antibiotic-resistant bacteria in the oral and intestinal flora of primates (Summers *et al*, 1993). In the first part of the study which involved human

volunteers, faecal samples were collected from human subjects. The amalgam status of each subject was not recorded, but the antibiotic history of each subject was noted. Subjects who had a high prevalence of mercury resistance in their intestinal flora and had not consumed antibiotics were also more likely to have a high number of strains resistant to antibiotics. Subjects with no detectable levels of mercury resistance were less likely to have strains resistant to antibiotics. In the second study, resistance to mercury and antibiotics were examined in the oral and intestinal flora of 6 adult monkeys prior to the installation of amalgam fillings, during the time they were in place and after replacement of the amalgam fillings with glass ionomer fillings. During this study, the faecal mercury concentrations were monitored. A statistically significant increase in the number of mercury-resistant bacteria during the 5 weeks following installation of the amalgam fillings and during the 5 weeks following replacement with glass ionomer fillings was observed. Some mercury-resistant Enterobacteriaceae and enterococci were also resistant to one or more antibiotics including ampicillin, tetracycline, streptomycin, kanamycin and chloramphenicol. Many of the mercury-resistant Gram-negative strains were able to transfer mercury and antibiotic resistance together to recipient strains suggesting that the loci for these resistances were genetically linked. This paper is important in that it not only supports evidence for a link between amalgam fillings and an increase in mercury and antibiotic resistance but is also the first report of mercury resistance in the oral streptococci. In addition, this work is very important as it illustrates that the commensal flora can act as a reservoir for antibiotic resistance genes, which under suitable conditions can be transferred to pathogenic organisms creating

antibiotic-resistant pathogens. Amalgam is the most common restorative material used worldwide and the potential impact of this source of mercury could have on the composition of the normal human flora is very large because unlike antibiotics, which are normally taken only occasionally, once the amalgam is in place, low level mercury is released for extended periods.

1.8 Antibiotics

1.8.1 The Discovery of Antibiotics

Antibiotics are low molecular mass substances that are produced as secondary metabolites by certain groups of micro-organisms, especially *Streptomyces*, *Bacillus* and the moulds, *Penicillium* and *Cephalosporium* (Brumfitt & Hamilton-Miller, 1988, Demain, 1992, Maplestone *et al*, 1992, Alderson *et al*, 1993). Most micro-organisms that produce antibiotics are resistant to the action of the antibiotic that they produce and it is generally not understood why (Maplestone *et al*, 1992, Stone & Williams, 1992). It may be helpful to study these mechanisms to gain a greater understanding of the cellular and molecular basis of resistance. The majority of organisms that produce antibiotics also form spores or resting structures. It is not fully known why these organisms produce antibiotics but production may give them some nutritional advantage in the habitat by antagonising the competition (Messenger & Turner, 1981, Stone & Williams, 1992). Alternatively, the antibiotic may act as some sort of hormone or signal molecule associated with

sporulation, dormancy or germination (Nakano & Zuber, 1990, Marahiel *et al*, 1993). Antibiotic synthesis may require as many as 30 separate enzymatic steps and retaining such a large number of genes in the genome has led to the conclusion that either the molecule and/or the process is important for the survival of these organisms in their natural habitat (Stone & Williams, 1992).

The earliest record of a substance produced by a mould that could apparently kill bacteria was made in 1896 by a medical student named Ernest Duchesne. However, his work was largely ignored. Then, in 1929, the Scottish physician Alexander Fleming observed inhibition of staphylococci on an agar plate contaminated by *Penicillium notatum*. The diffusible substance produced by the *Penicillium* killed many different kinds of bacteria and Fleming realised its potential usefulness in treating infections (Fleming, 1929, Brumfitt & Hamilton-Miller, 1988, Demain & Elander, 1999). However, the substance appeared very unstable and Fleming ceased working on the mould, publishing his last work on penicillin in 1931.

The development and clinical use of antibiotics began with the therapeutic application of the synthetic antimicrobials known as sulphonamides in 1935 (Domagk, 1935, Morris *et al*, 1998). Prontosil, a hydrochloride salt of 4'-sulphonamide-2,4-diaminoazobenzene, was originally part of a leather dye compound but had the ability to kill bacteria (Figure 1.8). The substance was relatively non-toxic and was found to be converted by the body to sulphanilamide.

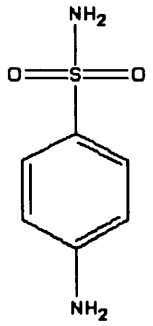

Figure 1.8: Chemical structure of Prontosil

World War II was an important impetus to the study of the chemotherapeutic value of penicillin. Penicillin was purified and injected into experimental animals, where it was found not only to cure infections but also to possess only low toxicity (Chain *et al*, 1940). With the successful testing of penicillin, the age of the use of naturally-occurring substances for the treatment of infections was born and the late 1940s and early 1950s saw the discovery and introduction into clinical practice of streptomycin, chloramphenicol and tetracycline (Schatz *et al*, 1944, Roberts, 1996, Chopra & Roberts, 2001, Stratton, 2002).

1.9 Properties of Clinically Useful Antibiotics

Antibiotics have a cidal (killing) effect or static (inhibitory) effect on a range of microbes. Desirable properties of a clinically useful antibiotic are as follows:

1. Non-toxic to the host, without undesirable side effects.
2. Non-allergenic to the host.
3. Harmless to the normal flora of the host.
4, Able to reach the infected region of the body at a desirable concentration.
5. Inexpensive and easy to produce.
6. Chemically stable.
7. Resistance is uncommon.

Table 1.1 summarizes the 5 basic sites of antibiotic activity. The most common site of antibiotic activity is interference with bacterial cell wall synthesis. Obviously, these agents are ineffective against wall-less organisms, such as *Mycoplasma*. Antibiotics such as the sulphonamides inhibit folic acid production. Polymyxins destroy cytoplasmic membranes of susceptible bacteria by producing a detergent-like activity resulting in leakage of the cell contents. Many antibiotics inhibit protein synthesis, while others inhibit DNA and RNA synthesis.

1.9.1 Antibiotic Resistance

Bacteria have been in existence for more than 3 billion years. Antibiotic resistance was well established long before the clinical use of antibiotics as a means of survival for bacteria exposed to antimicrobials produced by microbes in the surrounding environment (Holland, 1998). Surveillance of antimicrobial resistance began 60 years ago when the use of antimicrobial agents began and it was not long before microbiologists noted an increase in bacterial resistance to the antibiotics. For example, when penicillin was first introduced to the clinic, less than 1% of *Staph. aureus* were resistant. After a few years the proportion rose to 8% and at present more than 80% of clinically isolated *Staph. aureus* are resistant to penicillin (Ebrahim, 1995).

Antimicrobial resistant micro-organisms possess either intrinsic resistance or acquired resistance. Intrinsic resistance, or more accurately, insensitivity, is chromosomally-mediated and, typically, non-transferable (Huycke *et al*, 1998, Courvalin, 1996). Intrinsic resistance has been recognised since the early days of antibiotic therapy and refers to a trait that is present in all the members of a given bacterial genus or species. For example, Gram-negative bacteria, in particular *Enterobacteriaceae*, are naturally resistant to macrolides and resistant to glycopeptide antibiotics such as teicoplanin and vancomycin. This is due to the inability of the large molecules to permeate the outer membrane of the organism (Courvalin, 1996). Enterococci are intrinsically resistant to low-level aminoglycosides, such as

gentamicin, and the primary and secondary cephalosporins, while the lactic acid bacteria (*Lactobacillus*, *Leuconostoc* and *Pediococcus*) are resistant to glycopeptide antibiotics (Woodford *et al*, 1995). The motile enterococci (*Enterococcus gallinarum*, *Ent. casseliflavus* and *Ent. flavescens*) possess the *vanC* gene (chromosomal) and consequently show low-level vancomycin resistance, but teicoplanin susceptibility (Woodford *et al*, 1995).

Acquired resistance can either be indigenous, through mutations, or exogenous, due to the acquisition of foreign DNA from other organisms (Neu, 1992, Courvalin, 1996). Chromosomally-mediated resistance that arises through mutation can only be passed on vertically to daughter cells resulting in a gradual spread of resistance which is usually low-level (Ebrahim, 1995). This type of resistance is usually progressive, evolving from low level through intermediate to high level resistance (Roberts, 1998). For example, fluroquinolone resistance in *E. coli* requires several sequential mutations to reach a clinically relevant level of resistance (Levy, 1998). Penicillin-resistant *Streptococcus pneumoniae* emerged after gradual progression from reduced susceptibility to high-level resistance (Goldstein & Gerau, 1994, Jacobs, 1999). This highlights the importance of constant surveillance, where an increasing minimum inhibitory concentration (MIC) is a possible indicator of future resistance (Levy, 1998).

Acquisition of exogenous resistance occurs through the exchange of genetic material by transformation, transduction or conjugation (Figure 1.9)(Courvalin, 1996).

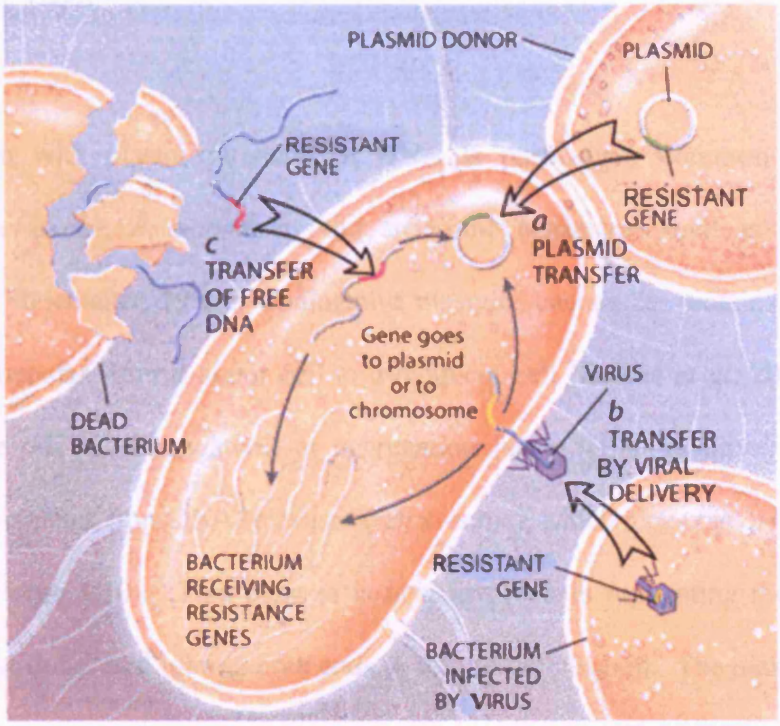

Figure 1.9: Gene transfer in bacteria

a) conjugation, b) transduction and c) transformation (Miller, 1998)

Transduction is limited to closely-related species as a high degree of specificity is required in the adsorption step in bacteriophage invasion (Courvalin, 1996). Similarly, transformation may be confined to intrageneric transfer (Courvalin, 1996).

In transformation, DNA fragments are transferred into the genome of a naturally-transformable recipient organism by homologous recombination (Spratt *et al*, 1992). Transformation accounts for the build up of mosaic genes responsible for penicillin resistance by the production of hybrid penicillin-binding

proteins in *S. pneumoniae*, *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Spratt *et al*, 1992).

Conjugation with plasmid transfer of DNA is particularly common among the *Enterobacteriaceae*, *Pseudomonas* and anaerobic organisms such as *Bacteroides* (Salyers & Shoemaker, 1996). Conjugative plasmids encode the proteins involved in their own transfer from a donor cell to a recipient cell (Burrus *et al*, 2002). These include the pili (Gram-negative) or aggregation factor (Gram-positive) needed for cell-to-cell contact, plus DNA relaxases (relaxosome), which nicks the *ori*T site of the plasmid (Figure 1.10). This enzyme is also involved in replicating the remaining strand in the donor and joining both ends to reform the plasmid. The nicked strand is transferred through the pili or mating pore and replicates in the recipient cell (Grohmann *et al*, 2003). Conjugative plasmids can efficiently transfer between Gram-positive or Gram-negative bacteria belonging to different genera but not between Gram-positive and Gram-negative organisms and vice versa (Courvalin, 1994). This is because their host range for replication is more narrow than that for transfer, meaning that although conjugation can occur, the DNA cannot replicate within the new host.

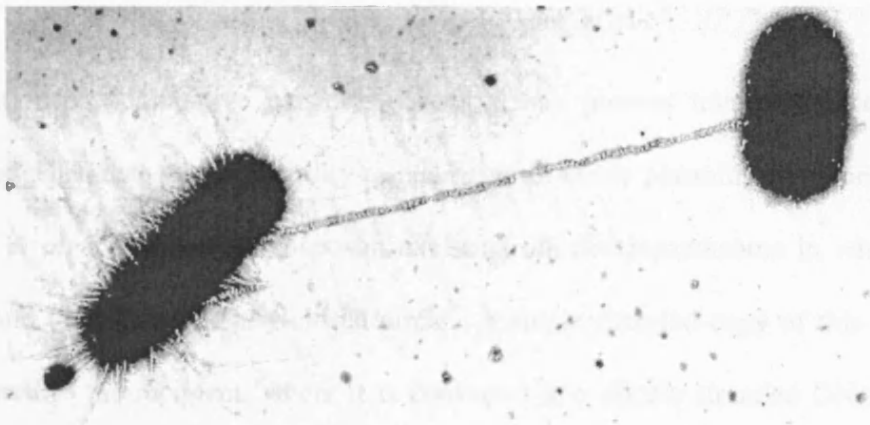

Figure 1.10: Conjugation between Gram-negative bacteria

Since the discovery of conjugative plasmids, chromosomal conjugative elements have been identified and found that, unlike conjugative plasmids, cannot be isolated as circular replicative molecules. Site-specific recombinases encoded by these elements promote their excision and integration (Burrus *et al*, 2002). These elements are known as conjugative transposons and can transpose both intracellularly and intercellularly. They contribute to the spread of antibiotic resistance genes in several clinically important groups of bacteria, including Gram-positive cocci, some *Bacteroides* species and certain Gram-negative bacteria (Speer *et al*, 1992, Salyers *et al*, 1995, Salyers & Shoemaker, 1996, Smith *et al*, 1998, Rowe-Magnus *et al*, 2002). The best-studied chromosomal conjugative transposon is *Tn916*, found in *Ent. faecalis*, which carries the *vanB* gene (Manganelli *et al*, 1995, Burrus *et al*, 2002). Conjugative transposons differ from conjugative plasmids in that the circular intermediate of a conjugative transposon does not replicate, at least in hosts so far investigated (Speer *et al*, 1992).

In addition to conjugative plasmids, bacteria may possess transposons, so-called jumping genes that have the ability to enter transmissible plasmids or chromosomes (Haren *et al*, 1999). The transposon excises from the chromosome in which it is found and forms a covalently-closed circle. A single stranded copy of this circle is transferred to the recipient, where it is converted into double stranded DNA, which integrates into the DNA of the new host (Salyers & Shoemaker, 1996).

Studies have shown that regions that flank resistance genes in transposons and plasmids of Gram-negative bacteria often show high similarity (Olsen, 1999). This realisation led to the discovery of integrons. Integrons are natural genetic engineering elements that are able to stockpile and express selectable genes. The definitive features of integrons are that they encode a site-specific integrase (IntI) and have a proximal primary recombination sequence site (*attI*)(Hall & Collis, 1998). The integrase mediates recombination between the *attI* site and a secondary target called an *attC* site. The 59bp *attC* sites are found on gene cassettes. These are discrete genetic elements that may exist as free, circular, non-replicating DNA molecules when moving from one cell to another but are normally found as linear sequences within a larger DNA molecule such as a plasmid or bacterial chromosome (Bennett, 1999). Gene cassettes normally contain a single gene of approximately 500-1000bp in length, which usually lacks a promotor. Once integrated with an integron, the gene is expressed by the integron's promotor (Rowe-Magnus & Mazel, 2001). Although integrons are unable to self-transpose they

are often found associated with transposons and conjugative plasmids that can serve as vehicles for the intra- and inter-species transfer of genetic material. Their impact on the development of multi-antibiotic resistance has been considerable (Rowe-Magnus *et al*, 2002).

1.9.2 Selective Pressure

The use of antibiotics in the clinic results in selective pressure, which may give rise to an increased incidence of antibiotic resistance. An increasing rate of antibiotic consumption in several countries has resulted in a steady rise in the prevalence of resistant strains (Austin *et al*, 1997). In some countries there are a bewildering variety of proprietary drugs which contain irrational mixtures of antibiotics and other preparations such as vitamins, steroids and stimulants. These mixtures could be regarded as similar to the growth promoters often used in animal husbandry (Greenhalgh, 1986). Brazil alone has 117 different brands of ampicillin and amoxycillin, while Indonesia markets more than 13,000 drug formulations (Ebrahim, 1995). Additionally, in certain countries such as Mexico, Spain, Taiwan and China, antibiotics can be bought cheaply over-the-counter without a prescription, which may result in increased antibiotic consumption and, more importantly, use of the wrong drug (Livermore, 1995).

Long-term antibiotic consumption leads to long-term selective pressure, which can result in an increased incidence of antibiotic resistance. This is seen in subjects

taking broad-spectrum antibiotics such as neutropaenic cancer patients and acne sufferers, who take fluoroquinolones and tetracyclines, respectively (Eady, 1998). Broad-spectrum agents act on a wide range of micro-organisms, resulting in the eradication of both pathogenic and commensal organisms. Surprisingly, the use of antibiotics by one person can affect other individuals in the immediate and extended environment. Studies have shown that the housemates of acne sufferers treated with antibiotics also had large numbers of drug-resistant flora on their skin (Miller *et al*, 1996).

Antibiotic resistance is becoming a great concern and the problem is impossible to reverse. Once resistance appears it is likely to decline slowly, if at all. There are no counter-selective measures against resistant bacteria and the slow loss of resistance is linked to poorly reversible genetic and environmental factors. Resistance is becoming a greater concern because there are no antibiotics to which resistance has not eventually appeared (Levy, 1997). Additionally, despite the intensive research that is being conducted by the pharmaceutical industry, only one new class of antimicrobials, the oxazolidinones, has been launched since 1972 (Livermore, 2003). The other newly introduced antimicrobials are permutations (analogues) of pre-existing compounds. Therefore, it is common to see resistance to these new antibiotics even before they have been introduced to the clinical environment. A global priority is to encourage the development of novel classes of antibiotics. However a major limitation is that it can cost up to $300 million to discover a new class of antibiotic (Levy, 1997).

At present there are very few ways in which the emergence of antibiotic resistance can be slowed and it is near impossible to decrease. One way is that the general public must be better educated and understand that antibiotics are not miracle drugs, and that treatment may fail and may even be harmful. In addition, prescribers need to remain up to date on diagnosis and treatment of infectious diseases and not succumb to patient pressure for antibiotics (Williams & Heymann, 1998).

1.10 Penicillin Resistance

1.10.1 The Penicillins

Penicillin is an example of a β-lactam antibiotic. The β-lactams are potent bactericidal agents of widespread clinical use and show low toxicity to eukaryotes. Antimicrobials belonging to this group include the penicillins (penems), cephalosporins (cephems), monobactams and carbapenems. Beta-lactam antibiotics have been in clinical use for more than 50 years and the susceptibility of various pathogenic bacteria to these agents has changed dramatically over the years due to their widespread and liberal usage (Kotra & Mobashery, 1998).

Beta-lactams inhibit the synthesis of peptidoglycan, a major polymer of the bacterial cell wall. The targets for β-lactam antibiotics are cell wall-synthesising enzymes, which, due to their ability to bind covalently radiolabelled penicillin, are known as

Penicillin-binding Proteins (PBP)(Blumberg & Strominger, 1974). PBPs are present in nearly all bacteria and vary from species to species in number, size, quantity and affinity for β-lactam antibiotics (Georgopapadakou, 1993). The functions of PBPs are diverse and include transpeptidase, transglycosylase and carboxypeptidase activities (Massova & Mobashery, 1998). The enzymes are localized non-randomly on the outer face of the cytoplasmic membrane and are anchored through short hydrophobic carboxy- or amino-terminal sequences (Georgopapadakou, 1993). All prokaryotes have between 2 and 4 essential PBPs and therefore the β-lactams have multiple targets. Inhibition of any of these enzymes can lead to cell lysis, death or growth arrest (Georgopapadakou, 1993).

1.10.2 Penicillin Resistance Mechanisms

Micro-organisms resistant to β-lactams exhibit either altered PBPs or produce β-lactamases (penicillinases). In addition, some Gram-negative organisms also exhibit altered outer membrane permeability (Philippon *et al*, 1989).

Altered PBPs are more commonly found in Gram-positive than Gram-negative bacteria (Georgopapadakou, 1993). The most common example of PBP-mediated resistance is methicillin-resistant *Staph. aureus* (MRSA) (Brumfitt & Hamilton-Miller, 1989). *Staphylococcus aureus* normally has five PBPs while methicillin-resistant organisms have an additional 78-kDa PBP, known as 2a or 2', which has a low affinity for β-lactams and catalyses a penicillin-insensitive

transpeptidation (de Jonge *et al*, 1992). This enzyme is encoded by a chromosomal gene known as *mecA* (Georgopapadakou, 1993). Other Gram-positive organisms such as *Staphylococcus epidermidis*, *S. pneumoniae*, viridans streptococci and enterococci possess altered PBPs (Georgopapadakou, 1993). Resistance to β-lactams in Gram-negative bacteria is not commonly associated with altered PBPs, probably due to the effectiveness of β-lactamases, coupled with reduced outer membrane permeability (Spratt, 1988).

Beta lactamases are the most common cause of bacterial resistance to β-lactam antibiotics (Livermore, 1995). The β-lactamases have been classified into 4 groups. Beta-lactamases belonging to groups A, C and D possess serine residues, which participate in the active site of the enzyme (Kotra & Mobashery, 1998). Class B β-lactamases are metalloenzymes and utilize zinc to disrupt the β-lactam ring (Livermore, 1995). Beta lactamases are encoded by genes found both chromosomally and, more commonly, on plasmids. Examples of these enzymes include SHV (sulphydryl variable), TEM (Temoniera) and PSE (*Pseudomonas*-specific enzyme). A range of β-lactam inhibitors have been developed such as clavulanate, sulbactam and tazobactam, which render resistant organisms sensitive (Sirot, 1995). However, β-lactamase-inhibitor combinations do not reduce the MIC greatly, when compared with organisms without the β-lactamase protein.

Extended-spectrum β-lactamases (ESBLs) are mutant enzymes which derive from TEM or SHV (Class A) enzymes. They confer variable levels of resistance to

cefotaxime, ceftazidime and other broad-spectrum cephalosporins and monobactams. They have no activity against cefamycins and carbapenems (Sirot, 1995). ESBLs have been detected in nearly all species of Enterobacteriaceae, such as *E. coli* and *Enterobacter aerogenes* but are more common in *K. pneumoniae* (Jacoby, 1994, Sirot, 1995). Most EBSLs are found on plasmids and allows resistance to spread readily to other pathogens (Sirot, 1995).

1.10.3 Oral Flora and β-lactam Resistance

Beta-lactamase producing strains of *Haemophilus*, *Actinomyces*, *Veillonella*, *Lactobacillus*, *Clostridium*, *Peptostreptococcus*, *Streptococcus*, *Bacteroides*, *Neisseria*, *Moraxella*, *Eikenella* and *Capnocytophaga* have been isolated from the oral flora of humans (Slots, 1979, Moore *et al*, 1984, Wasfy *et al*, 1986, Lacroix & Walker, 1992, Roberts, 1998, Packer *et al*, 1999).

1.11 Vancomycin Resistance

1.11.1 The Glycopeptides

Vancomycin is a glycopeptide antibiotic, isolated from *Streptomyces orientalis* and, along with teicoplanin, inhibits cell wall synthesis in Gram-positive bacteria by interacting with the terminal D-alanyl-D-alanine (D-Ala-D-Ala) group of the pentapeptide side chains of peptidoglycan precursors (Reynolds, 1989).

This interaction prevents the transglycosylation and transpeptidation reactions required for polymerisation of peptidoglycan. Glycopeptides are large molecules; vancomycin has a molecular mass of 1,448 and teicoplanin is 1,900; and thus are unable to permeate the outer-membrane of Gram-negative organisms and reach the target site. Therefore, nearly all Gram-negative organisms are resistant to glycopeptide action, while nearly all Gram-positive organisms are susceptible (Woodford *et al*, 1995). Since the introduction of vancomycin in the 1950's the glycopeptide class of antibiotics has been used to treat diseases caused by Gram-positive organisms possessing intrinsic resistance to other agents, as in *Clostridium difficile* and *Corynebacterium jeikeium*, or acquired resistance, such as MRSA.

1.11.2 Vancomycin Resistance Mechanisms

The introduction of new antibiotics into clinical use is usually followed by the fairly rapid emergence of bacterial resistance. However, isolates resistant to vancomycin were not found until almost 30 years after the introduction of vancomycin to the clinic (Woodford *et al*, 1995). In addition, resistance to vancomycin was reported only rarely and appeared to have little clinical significance (Woodford *et al*, 1995). However, in 1988 there were two reports of plasmid-mediated, high-level resistance to both vancomycin and teicoplanin in *Enterococcus* species (Woodford *et al*, 1995). These and subsequent reports alerted microbiologists to the possibility of glycopeptide resistance in enterococci and many laboratories began screening for it.

Recent studies by the National Nosocomial Infections Surveillance programme of the Centers for Disease Control and Prevention (CDC) found that up to 10% of hospital-acquired enterococci isolated from patients in intensive care units (ICUs) were resistant to vancomycin. Treatment of these infections is usually difficult since these organisms are also often resistant to other agents such as β lactams and macrolides (Bonafede & Rice, 1997).

Enterococci are increasingly prominent nosocomial pathogens and, due to the increasing number of vancomycin resistant organisms isolated, many laboratories are studying vancomycin resistance. To date, 5 glycopeptide resistance phenotypes have been described in enterococci (Woodford, 2001). The two primary clinical important phenotypes are VanA and VanB. VanA strains express high-level vancomycin and teicoplanin resistance, while VanB strains express low-level vancomycin resistance and susceptibility to teicoplanin (Arthur & Courvalin, 1993). The apparent susceptibility to teicoplanin is due to teicoplanin being a poor inducer of the VanB operon. VanC confers low-level resistance to vancomycin and is an intrinsic property of most isolates of *Ent. casseliflavus*, *Ent. flavescens* and *Ent. gallinarum* (Woodford *et al*, 1995, Woodford, 1998).

1.11.3 Oral Flora and Vancomycin Resistance

Resistance to vancomycin is rarely encountered in viridans streptococci. Resistance was first reported in Slovakia in 1996, while vancomycin resistance in enterococci had been reported 8 years previously (Uttley *et al*, 1988, Krčméry *et al*, 1996a). The vancomycin-resistant *S. mitis* displayed low-level resistance to vancomycin (MIC 16-32mg/l) and was sensitive to teicoplanin, suggesting that the organism had an enterococcal *vanB* gene. Since then, studies have shown *in vitro* transfer of the *vanA* gene from *Ent. faecium* to *S. sanguinis*, resulting in high-level vancomycin resistance (MIC 128mg/l)(Leclercq *et al*, 1989).

1.12 Erythromycin Resistance

1.12.1 The Macrolides

McGuire and coworkers discovered erythromycin in 1952 when studying the metabolic products of a strain of *Saccharopolyspora erythaea* (previously known as *Streptomyces erythreus*), which had been collected from a soil sample from the Phillippine Archipelago (Roberts *et al*, 1999). Erythromycin can be either bacteriostatic or bacteriocidal depending on the drug concentration, organism susceptibility, organism growth rate and size of inoculum. Erythromycin belongs to the macrolide class of antibiotics, which along with the lincosamides and

streptogramins inhibit protein synthesis by binding to the 50S bacterial ribosomal subunit (Weisblum, 1995, Roberts, 1998). Although all 3 classes are structurally unrelated, all share overlapping binding sites on the 50S ribosomal subunit. The newer derivatives of macrolides possess both Gram-negative and Gram-positive antimicrobial activity, although the older derivatives such as erythromycin only show activity against Gram-positive bacteria and certain Gram-negative genera such as *Neisseria*, *Haemophilus* and *Bacteroides* (Roberts *et al*, 1999). In addition, erythromycin is active against mycobacterial infections (Piddock, 1998).

1.12.2 Erythromycin Resistance Mechanisms

Bacteria possess a number of different mechanisms of resistance to macrolides, including efflux, drug inactivation and target site alteration (Roberts, 1998). Ribosomal mutation is the most common mechanism and this is carried out by a rRNA methylase that post-translationally modifies an adenine residue at position 2058 of the 23S rRNA (Eady *et al*, 1990, Roberts, 1998). This leads to a conformational change in the ribosome, resulting in cross resistance and prevents both macrolides, lincosamides and type B streptogramins (MLS_B) from binding to the 50S ribosomal subunit (Leclercq & Courvalin, 1991a, 1991b, Roberts *et al*, 1999).

Ribosomal RNA methylases are encoded by the *erm* genes (erythromycin resistant methylases) and to date more than 30 *erm* genes have been isolated (Roberts, 1998). They have been isolated from a wide variety of species including a number of

different Gram-positive and a limited number of Gram-negative organisms (Table 1.2)(Roberts *et al*, 1999). The genes have been grouped into families based on their DNA and amino acid homology and every new gene isolated from a different species is given a new letter designation (Leclercq & Courvalin, 1991a, 1991b). The majority of *erm* genes are highly related to *ermB* and *ermAM* (Roberts, 1998). The *ermB* group was first described in streptococci and has now been isolated from oral bacteria such as *A. actinomycetemcomitans*, *Treponema denticola* and *Fusobacterium*, *Haemophilus*, *Peptostreptococcus*, *Porphyromonas* and *Vellionella* species (Roberts, 1998). The ErmC methylase has been found in both Gram-negative (*A. actinomycetemcomitans* and *Neisseria* species) and Gram-positive organisms (*Lactobacillus* and *Streptococcus* species)(Roberts *et al*, 1999). The *ermA* gene has been found in staphylococci and *ermQ* in *A. actinomycetemcomitans* (Weisblum, 1995, Roberts *et al*, 1999). The *erm* genes are often chromosomal and associated with either transposons or conjugative transposons (Roberts, 1998). They are often associated with other antibiotic resistance genes, especially tetracycline resistance genes (Roberts *et al*, 1999). The *ermB* gene is often linked with the *tetM* gene, while the *ermF* gene is often linked with the *tetQ* gene (Roberts *et al*, 1999).

The efflux mechanism of resistance, designated the M phenotype, causes resistance to 14-membered (roxithromycin, clarithromycin, oleandomycin, erythromycin and dirithromycin) and 15-membered (azithromycin) macrolides (Leclerq, 2002). In *S. pyogenes*, the *mefA* (macrolide efflux) gene and in *S. pneumoniae*, the *mefE* gene

encodes proteins involved in efflux (Roberts, 1998). In *Staph. aureus*, the *msrA* gene, and in *Staph. epidermidis*, the *mreA* gene, encode proteins involved in efflux (Eady *et al*, 1990, Clancy *et al*, 1997).

Drug inactivation is a mechanism less commonly observed in organisms, although it has been detected in *E. coli*, *Staph. aureus*, lactobacilli and coagulase-negative staphylococci (Leclerq & Courvalin, 1991a, 1991b). Unlike target-site alternation, enzymatic modification of MLS_B antibiotics is highly specific (Roberts, 1998). For example, an organism possessing the erythromycin esterase confers resistance to erythromycin and other 14-membered macrolides only.

1.12.3 Oral Flora and Macrolide Resistance

Numerous laboratories around the world have looked for macrolide resistance genes in the oral flora, especially in viridans streptococci. To date, genes encoding efflux proteins (*mef*) and ribosomal methylases (*erm*) have been found. *Erm* genes have been found in *S. oralis* and *S. mitis* (Table 1.2)(Poutanen *et al*, 1999, Ono *et al*, 2000). *Prevotella*, *Porphyromonas* and *Bacteroides* have been found to possess *ermF* and strains of the '*S. milleri*' group have the *ermB* gene (Arzese *et al*, 2000, Jacobs *et al*, 2001, Chung *et al*, 2002). *MefE* is found in *S. oralis*, *S. mitis* and *S. salivarius*, while *mefA* is found in strains of *S. oralis* (Arpin *et al*, 1999, Ono *et al*, 2000).

1.13 Tetracycline Resistance

1.13.1 The Tetracyclines

The development of the tetracycline antibiotics was the result of systematic screening of soil specimens collected from many parts of the world for antibiotic-producing microorganisms (Brumfitt & Hamilton-Miller, 1988). Chlortetracycline, obtained from *Streptomyces aureofaciens*, was introduced in 1948, and in 1952, a third member, tetracycline, was produced semi-synthetically from chlortetracycline (Roberts, 1996, Chopra & Roberts, 2001). Tetracyclines are broad-spectrum antimicrobials that are active against a wide range of Gram-positive and Gram-negative bacteria, cell wall-free mycoplasmas, chlamydiae, rickettsiae and protozoan parasites (Roberts, 1998, Chopra & Roberts, 2001). Due to their broad spectrum of activity, relative safety and low cost, tetracyclines have been used widely throughout the world and are the second most commonly used antibiotic after penicillins (Roberts, 1994, 1998). Tetracyclines also possess non-antimicrobial properties including anti-inflammatory and immunosuppressive properties, antibody production suppression, reduction in leucocyte and neutrophil chemotaxis, reduction in phagocytic function of polymorphonuclear leucocytes (PMN), inhibition of lipase and collagenase activity and anti-tumour activity (Roberts, 1996). These additional properties have led to an increase in the use of tetracyclines, resulting in an increase in the overall consumption of the antibiotic and greater exposure of the normal flora to the antimicrobial (Roberts, 1998).

Tetracyclines gain access into the bacterial cell wall by passive diffusion through hydrophilic pores in the outer cell membrane and then through the inner cytoplasmic membrane by energy-dependent transport (Roberts, 1996). The tetracyclines act by blocking the binding of aminoacyl tRNA to the acceptor site (A site) on the ribosome.

1.13.2 Tetracycline Resistance Mechanisms

During the past 20 years resistance to tetracycline has limited their use (Roberts, 1994). Resistance to tetracycline is primarily due to the acquisition of Tet determinants rather than to mutation of existing chromosomal genes (Roberts, 1994). To date, 29 tetracycline resistance determinants (Tet) and 3 oxytetracycline resistance determinants (Otr) have been described and characterized (Chopra & Roberts, 2001). There is no inherent difference between a tetracycline and oxytetracycline resistance gene (Chopra & Roberts, 2001). The oxytetracycline genes were first identified in oxytetracycline-producing organisms and thus nomenclature reflects the organisms first shown to carry the particular gene (Chopra & Roberts, 2001). Of the 29 determinants, 14 are often associated with plasmids, while others are found on the chromosome (Table 1.3). The most widespread tet^r determinant is *tetB* which has been identified in 20 Gram-negative genera. The *tetB* gene is found on conjugative plasmids of oral bacteria such as *Actinobacillus*, *Pasteurella, Providencia, Treponema* and most *Haemophilus* species (Table 1.4)(Roberts, 1998). TetM is the second most frequently described

tet^r determinant and has been identified in 28 different genera and in a large number of different species (Roberts, 1998). TetM determinants are associated with conjugative elements found in both Gram-positive peptostreptococci and streptococci and Gram-negative *Fusobacterium* and *Veillonella* (Roberts, 1998)(Tables 1.4 & 1.5). Many tetracycline-resistant bacteria contain two tet determinants and often the MIC of tetracycline towards these organisms is greater than those with only one tet^r determinant (Rodriguez-Avial *et al*, 2003).

To date, three different mechanisms of tetracycline resistance have been studied:

1. Energy-dependent efflux of tetracycline by proteins inserted into the cytoplasmic membrane.
2. Protection of the bacterial ribosome from the action of tetracycline.
3. Enzymatic alteration and inactivation of tetracycline.

The tet^r determinants associated with these mechanisms are summarised in Table 1.6.

Efflux proteins have been the best studied of the tet^r determinants and all these genes code for energy-dependent membrane-associated proteins which export tetracycline out of the cell (Roberts, 1994). The protein is approximately 46kDa consisting of 12 hydrophilic sequences. The efflux proteins exchange a protein for a tetracycline-cation complex and are antiporter systems (Roberts, 1994). The efflux proteins have amino acid and protein structure similarities with other efflux proteins

involved in multiple-drug resistance and resistance to quaternary ammounium compounds (QAC), chloramphenicol and quinolones.

The ribosomal protection mechanism of tetracycline resistance was first discovered in streptococci (Burdett, 1986). These are cytoplasmic proteins that protect the ribsosome from the action of tetracycline and confer resistance to doxycycline and minocycline. They confer a wider spectrum of resistance to tetracyclines than is seen with bacteria that carry tetracycline efflux proteins (Chopra & Roberts, 2001). Ribosomal protection proteins have been sequenced and have amino acid sequences similar to elongation factor G (EF-G) and elongation factor Tu (EF-Tu) (Taylor & Chau, 1986). The proteins show a ribosome-dependent GTPase (Chopra & Roberts, 2001).

To date, examples of enzymatically mediated resistance to tetracycline include TetX, Tet34 and Tet37 (Chopra & Roberts, 2001). The presence of TetX in Bacteroides is unusual because the protein is not operational in the absence of oxygen (Speer *et al*, 1991). Bacteroides are obligate anaerobes and therefore the clinical significance of TetX is questionable.

1.13.3 Oral Flora and Tetracycline Resistance

Tetracycline antibiotics are frequently used as adjuncts to conventional periodontal therapy and thus many groups have studied the incidence of *tet* genes in periodontal pathogens. *Mitsuokella multiacidus*, *Porphyromonas*, *Prevotella* and *Bacteroides* species have been found to contain the *tetQ* gene (Leng *et al*, 1997, Chung *et al*, 2002). *TetM* has been found in *Actinomyces*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Lactobacillus*, *Peptostreptococcus*, *Veillonella*, *Ureaplasma* and *Streptococcus* (including '*S. milleri*', *S. gordonii*, *S. mitis*, *S. oralis*, *S. parasanguinis* and *S. sanguinis*), (Tables 1.4 & 1.5) (Lacroix & Walker, 1995, Roberts, 1996).

1.14 Metronidazole Resistance

1.14.1 The 5-nitroimidazoles

Metronidazole and other 5-nitroimidazole drugs are valuable agents in the treatment of several protozoal and anaerobic bacterial infections (Freeman *et al*, 1997, Piddock, 1998, Samuelson, 1999). However, compared to other classes of antibiotics, very little is known about the nitroimidazole compounds. Metronidazole enters the cell by diffusion and the nitro-group is reduced to a radical anion, while the released nitroso and hydroxylamine derivatives interfere with DNA synthesis and degrade

existing DNA, resulting in cell death (Edwards, 1993). The process occurs at a low oxidation-reduction potential found in anaerobic conditions and the reduction is due to a ferredoxin-linked hydrogenase (Church *et al*, 1996). Metronidazole activity decreases in the presence of oxygen, often resulting in the isolation of apparently metronidazole-resistant anaerobes but when these organisms are further characterised, it is shown to be the consequence of an insufficiently anaerobic atmosphere (Piddock, 1998).

1.14.2 Metronidazole Resistance Mechanisms

Resistance to metronidazole is still rare, but is sometimes seen in clinical isolates especially *Bacteroides* species (Greenstein, 1993). Only a few metronidazole resistance genes have been cloned and characterised and these have been found to be located on both chromosomes and plasmids. They have been shown to be transferable by conjugation or by transformation, leading to the possibility of metronidazole resistance becoming more widespread in the future (Smith *et al*, 1998). The genes *nimA*, *nimB*, *nimC* and *nimD* have found to confer moderate to high-level metronidazole resistance in colonic *Bacteroides* species (Piddock, 1998). The *nim* genes are likely to code for a 5-nitroimidazole reductase which enzymatically reduces the 5-nitroimidazole to a 5-amino derivative (Roberts, 1998).

1.14.3 Oral Flora and Metronidazole Resistance

Metronidazole-resistant *A. actinomycetenemcomitans*, *B. fragilis* and *Fusobacterium nucleatum* have been isolated from subgingival plaque (Van Winckelhoff *et al*, 2000).

1.15 Aims of the study

There were several aims to the study. As very little information was available on mercury sensitivity testing, the first aim was to determine the most suitable agar and concentration of mercuric chloride to use in this project. The second aim was to determine whether children with fillings harboured a higher proportion of mercury-resistant bacteria in their oral flora than children without amalgam fillings. This was achieved through both a cross-sectional and longitudinal study. A further aim of the longitudinal study was to determine whether placement of mercury amalgam fillings in children's teeth resulted in an increase in oral bacteria resistant to penicillin, ampicillin, erythromycin, vancomycin, tetracycline or metronidazole. The third aim was to identify the mercury-resistant bacteria and determine whether there were differences in the types of organism isolated from individuals with and without amalgam fillings. An additional objective was to investigate whether the mercury-resistant organisms were also resistant to 6 antibiotics and to determine whether there were differences between the non-amalgam and amalgam individuals. Finally, the mercury-resistant bacteria were screened using molecular techniques to

determine whether they possessed the *merA* gene. A selection of the amplified genes were sequenced and compared to the *B. cereus* RC607 *merA* gene using the BLAST database.

Mechanism	Bacteriostatic or Bacteriocidal	Comments	Examples
Damage cell membrane allowing contents to leak out	Bacteriocidal	High toxicity to animals and humans. Topical use only	Polymyxin
Inhibitors of bacterial cell wall synthesis	Bacteriocidal	Animals and human do not have cell walls, so they are not affected	Penicllins Cephalosporins Bacitracin (topical) Glycopeptides
Inhibitors of folic acid synthesis which is needed for RNA and DNA synthesis	Bacteriostatic	Animals and humans get folic acid from diet	Sulphonamides Trimethoprim Cotrimoxazole
Inhibitors of protein synthesis	Bacteriocidal/ Bacteriostatic	Eukaryotic and prokaryotic ribosomes are different. High doses of the drug can affect animals and humans	Tetracyclines Aminoglycosides Chloramphenicol Macrolides
Inhibitors of DNA function	Bacteriocidal	Drugs used affect bacterial cells more than animal or human cells	Quinolones Metronidazole Rifampicin

Table 1.1: Sites of antibiotic activity

Gram-positive, mycobacteria		Gram-negative	
Bacterium	*erm* gene(s)	Bacterium	*erm* gene(s)
Actinomyces	C, F	*Acinetobacter*	B
Aerococcus	B	***Actinobacillus***	A, B, C, F, Q
Arcanobacterium	B, X	***Bacteroides***	A, B, C, F, G, 35
Arthrobacter	R	*Enterobacter*	B
Bacillus	B, D, C, G, 34	*Escherichia*	B
Clostridium	F, Q	***Eubacterium***	B, C,F
Corynebacterium	B, C, X	***Fusobacterium***	B, F
Enterococcus	B, C, F	***Haemophilus***	C, F
Gardnerella	F	*Klebsiella*	B
Lactobacillus	B, C, G, T	***Neisseria***	B, C, F
Micrococcus	B, C, 36	*Pantoea*	B
Micromonospora	W	***Porphyromonas***	B, F, G
Mobiluncus	F	***Prevotella***	A, C, F, G
Mycobacterium	37, 38, 39	*Proteus*	B
Pediococcus	B	*Pseudomonas*	B
Peptostreptococcus	A, B, C, F	*Selenomonas*	F
Propionibacterium	X	*Serratia*	B
Rothia	B	***Treponema***	F
Staphylococcus	A, B, C, F, Q, Y, 33	***Veillonella***	F
Streptococcus	A, B, C, F, Q	***Wolinella***	B, C, F, Q
Streptomyces	E, H, I, N, O, S, U, V, Z, 30, 31, 32		

Table 1.2
Distribution of *erm* gGenes among Gram-positive Bacteria, Mycobacteria and Gram-negative Bacteria
Species in bold are part of the human oral flora
(Modified from Roberts *et al*, 1999)

Plamid	Chromosome
Tet A-E	Tet B (rare)
Tet X	-
Tet G, H	-
Tet K	Tet K
Tet L	Tet L (rare)
Tet M (rare)	Tet M
Tet O	Tet O
Tet P	-
-	Tet Q
Tet S	-
-	Tet W
-	Tet 32
-	Otr A-C

Table 1.3
Location of the tetracycline resistance (Tet) determinants
(Modified from Roberts, 1994, 1996 and Villedieu *et al*, 2003)

Efflux		Ribosomal protection and/or efflux	
Bacterium	Tet determinant(s)	Bacterium	Tet determinant(s)
Actinobacillus	Tet A, B, H, L, O	*Acinetobacter*	Tet A, B, H, M, 39
Aeromonas	Tet A, B, D, E, 31	***Bacteroides***	Tet A, M, Q, X, 36
Agrobacterium	Tet 30	***Butyrivibrio***	Tet O, W
Alcaligenes	Tet E	***Camplyobacter***	Tet O
Alteromonas	Tet D	***Capnocytophaga***	Tet Q
Brevundimonas	Tet B	***Eikenella***	Tet M
Citrobacter	Tet A, B, C, D	***Fusobacterium***	Tet L, M, W
Edwardsiella	Tet A, D	***Haemophilus***	Tet A, B, K, M
Enterobacter	Tet B, C, D, M	***Kingella***	Tet M
Erwinia	Tet B	*Megasphaera*	Tet O, W
Escherichia	Tet A, B, C, D, E, G, M, I, Y	***Mitsuokella***	Tet M, Q, W
Eubacterium	Tet K	***Neisseria***	Tet B, M, Q, O, W
Francisella	Tet C	***Pasteurella***	Tet B, D, H, G, M
Klebsiella	Tet A, B, C, D, M	***Porphyromonas***	Tet Q, W
Mannheimia	Tet B, G, H	***Prevotella***	Tet M, Q, W
Moraxella	Tet B, H	*Selenomonas*	Tet Q, W
Morganella	Tet L	***Veillonella***	Tet A, L, M, Q, S, W
Pantoea	Tet B	*Vibrio*	Tet A, B, C, D, E, G, M, 34, 35
Pasteurella	Tet B, D, H		
Photobacterium	Tet B, D, M, Y		
Plesiomonas	Tet A, B, D, J		
Proteus	Tet A, B, C, J		
Providencia	Tet B, E, I		
Pseudomonas	Tet A, C, E, G, M, 34		
Ralstonia	Tet M		
Salmonella	Tet A, B, C, D, G, L		
Serratia	Tet A, B, C, E, 34		
Shigella	Tet A, B, C, D		
Stenotrophomonas	Tet 35		
Treponema	Tet B		
Yersinia	Tet B		
Vibrio	Tet A, B, C, D, E, G		

Table 1.4
Distribution of tetracycline resistance (Tet) determinants among Gram-negative bacteria.
Species in bold are part of the human oral flora
(Modified from Roberts, 1994, 1996, Chopra & Roberts, 2001, Villedieu *et al*, 2003)

Efflux		Ribosomal protection and/or efflux	
Bacterium	Tet determinant(s)	Bacterium	Tet determinant(s)
Nocardia	Tet K	***Abiotrophia***	Tet M
		Actinomyces	Tet L, M, W
		Aerococcus	Tet M, O
		Bacillus	Tet K, L, M, W
		Bacterionema	Tet M
		Bifidobacterium	Tet M, W
		Clostridium	Tet K, L, M, P, Q, 32
		Corynebacterium	Tet M, Z, 33
		Eubacterium	Tet K, M, Q
		Gardnerella	Tet M, Q
		Gemella	Tet M
		Lactobacillus	Tet O, Q, S, W
		Listeria	Tet K, L, M, S
		Mobiluncus	Tet O, Q
		Mycobacterium	Tet K, L, M, V, Otr A, B, C
		Mycoplasma	Tet M
		Peptostreptococcus	Tet K, L, M, O, Q
		Staphylococcus	Tet K, L, M, O, U, W, 38
		Streptococcus	Tet K, L, M, O, Q, T, W
		Streptomyces	Tet K, L, M, W, Otr A, B, C, tcr3
		Ureaplasma	Tet M

Table 1.5
Distribution of tetracycline resistance determinants among Gram-positive bacteria, mycobacteria, *Mycoplasma*, *Nocardia*, *Streptomyces* and *Ureaplasma*
Species in bold are part of the human oral flora
(Modified from Roberts, 1994, 1996, Chopra & Roberts, 2001, Villedieu *et al*, 2003)

Efflux	Ribosomal	Enzymatic	Unknown
Tet A-E	Tet M	Tet X	Tet U
Tet G-J	Tet O	Tet 34	
Tet K-L	Tet P(B)[a]	Tet 37	
Tet P(A)[a]	Tet Q		
Tet U-V	Tet S		
Tet Y-Z	Tet T		
	Tet W		
Tet 30-31[b]	Tet[c]		
Tet 33			
Tet 35			
Tet 38-39			
Otr B-C	Tet 32		
Tcr 3[c]	Tet 36		
	Otr A		

Table 1.6
Classification of tetracycline resistance determinants according to their mechanism of resistance
(Modified from Roberts, 1994, 1996, Chopra & Roberts, 2001, Villedieu *et al*, 2003)

[a] Tet P(A) and Tet P(B) are counted as one gene
[b] First numbered genes
[c] This gene has not been given a new designation

Chapter Two

Materials and Methods

2.0 Materials and Methods

2.1 Microbiological Techniques

2.1.1 Bacteriological Agar

The bacteriological agar was purchased from Oxoid (Basingstoke, Hampshire, UK), with the exception of Mitis Salivarius agar, which was supplied by Difco Ltd (Beckton Dickinson, Cowley, Oxfordshire, UK). All agars were made according to the manufacturer's instructions and autoclaved in glass Duran bottles (Jencons, Leighton Buzzard, Bedfordshire, UK). Before pouring, the agar was allowed to cool to 50°C in a waterbath.

2.1.2 Bacteriological Broth

The Brain Heart Infusion and Tryptone Soya broths were purchased in powder form from Oxoid and made according to the manufacturers instructions.

2.1.3 Ringers Solution

Quarter-strength Ringers solution was obtained in tablet form from Oxoid and made according to the manufacturers instructions.

2.1.4 Antibiotics

The antibiotics, with the exception of gentamicin, were obtained in powder form from Sigma-Aldrich (Poole, Dorset, UK). Gentamicin was obtained in ampoules from Sigma-Aldrich.

The gentamicin, erythromycin, tetracycline, and kanamycin used were not 100% pure and had been assayed by the manufacturer to determine the actual amount of biologically active drug present. The vancomycin used had a potency greater than 100%. This is because vancomycin powder is now produced in a purer form than the reference standard powder initially characterised many years ago. The formula below was used to determine the weight of powder required to prepare stock solutions of 10,000mg/litre (Equation 2.1).

$$W = \frac{1000}{P} \times V \times C$$

P = Potency given by manufacturer in relation to base
V = Desired volume (ml) stock solution
C = Final concentration of solution (multiples of 1000)
W = Weight (mg) of antibiotic to be dissolved in V

Equation 2.1: Preparation of Antibiotic Stock Solutions

The preparation and storage of the antibiotics are shown in Table 2.1.

2.1.5 Phosphate Buffer

The phosphate buffer was prepared by mixing stock buffer A (KH_2PO_4, monobasic potassium phosphate)(Sigma-Aldrich) with stock buffer B (K_2HPO_4, dibasic potassium phosphate)(Sigma-Aldrich).

2.1.5.1 Stock Buffer A, KH_2PO_4 (0.2M)

27.22g	monobasic potassium phosphate (anhydrous, KH_2PO_4)
200ml	DD-H_2O

This was brought up to 1000ml with distilled water and autoclaved at 121°C for 15 minutes. This buffer was stored for 1 year at 2 - 8°C.

2.1.5.2 Stock Buffer B, K_2HPO_4 (0.2M)

45.64g	dibasic potassium phosphate ($K_2HPO_4.3H_2O$)
200ml	DD-H_2O

Distilled water was added to bring the solution up to 1000ml and autoclaved at 121°C for 15 minutes. This buffer was stored at 2 - 8°C for up to 1 year.

2.1.5.3 Phosphate Buffer, pH8.0 (0.1M)

To prepare the 0.1M phosphate buffer (pH8.0), 2.65ml of stock buffer A was mixed with 47.35ml stock buffer B and brought up to 100ml by adding distilled water. The pH was checked with a pH meter and adjusted as required with 1M sodium hydroxide (NaOH) or 0.1M phosphoric acid (H_3PO_4). Autoclaving can alter the pH, so the buffer was sterilised by filtration. Finally, the buffer was stored at 25°C for up to 1 year.

2.1.6 Mercuric Chloride

The mercuric chloride used in this study was obtained from Sigma-Aldrich. A 0.1M stock solution was prepared by dissolving the mercuric chloride powder in sterile distilled water. The stock solution was stored in a plastic universal container (Sarstedt, Leicester, UK) wrapped in aluminium foil to protect from sunlight and was discarded if not used on the day on which it was prepared.

2.1.7 Control Organisms

The mercury-resistant organisms used in this study were *Staph. aureus* NCTC 50581, *Ent. faecium* 664 1H1, *Enterococcus* CE13 and *Bacillus cereus* RC607. The former was obtained from the Health Protection Agency (HPA)(formerly known as the Central Public Health Laboratory, Colindale, London, UK) and the enterococci and bacillus strains were obtained from Professor Anne Summers, University of Georgia, Athens, Georgia, USA. The

enterococci were isolated from monkey gingiva, whereas the mercury-resistant *B. cereus* was isolated from Boston Harbour, Massachusetts, USA (Mahler *et al*, 1986, Summers *et al*, 1993). These mercury-resistant organisms were maintained by weekly subculturing on Mueller-Hinton (MH) agar containing 20μM $HgCl_2$. Professor Summers also provided a mercury-sensitive *S. mitis* strain, 606 4T1. The mercury-sensitive *Staph.. aureus* used in this study was *Staph. aureus* 8325-4, obtained from Dr Sean Nair (Department of Oral Surgery, Eastman Dental Institute). This organism and NCTC 50581 are genetically identical, except that the mercury-sensitive strain lacks the plasmid pI258 that harbours the mercury operon. The mercury-sensitive organisms were subcultured every 7 days on Columbia agar containing 5% defibrinated horse blood (CBA)(E&O Laboratories, Bonnybridge, Stirlingshire, UK).

The antibiotic-sensitive organisms, *E. coli* NCTC 10418 and *Staph. aureus* NCTC 6571 (Oxford *Staphylococcus*) were used to control the antibiotic breakpoint plates used in Chapters 4 and 5. These strains were obtained from the HPA and subcultured weekly onto CBA.

2.1.8 10% Glycerol-BHI Frozen Stocks

All the mercury- and antibiotic-resistant strains isolated from the study were frozen at -70°C in a 10% glycerol-BHI solution. The glycerol was bought from Sigma-Aldrich.

2.2 Bacterial Identification

The chemicals in this section were purchased from Sigma-Aldrich.

Table 2.2 shows the tests used to identify the mercury-resistant bacteria to the genus level.

2.2.1 Oxidase Reagent (Cytochrome c Oxidase Activity)

A fresh solution of the reagent was prepared each time of use by adding a loopful of N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride to 3ml sterile distilled water in a bijou container (Sarstedt). To test for oxidase activity a Q tip was immersed in the indicator solution and the excess solution removed by pressing the tip against the side of the bijou. The test colony was touched with the Q tip and the appearance of a dark purple colour on the Q tip within 30 seconds indicated a positive reaction.

2.2.2 Catalase Test

Catalase activity was detected using 3% hydrogen peroxide solution and a capillary tube. The presence of gas bubbles (O_2) indicated catalase activity.

2.2.3 Streptococcal Identification

2.2.3.1 Biochemical Method

TES Buffer

12.56g	N-tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid (TES)
1000ml	DD-H_2O

The pH was adjusted to pH7.5 with sodium hydroxide (NaOH) or hydrochloric acid (HCl) and the TES buffer was stored at 4°C.

Fermentation Tests

Carbohydrate Fermentation

The carbohydrate fermentation reactions were determined with a basal medium composed of Purple Broth Base and Thioglycollate medium (without dextrose or indicator)(Difco Ltd) with carbohydrates added at a concentration of 1% w/v:

16g	Purple Broth Base, basal medium
24g	Thioglycollate medium (without dextrose and indicator)

Distilled water was added to make a solution of 1000 ml and this was divided into ten 100ml aliquots. The nine sugars tested were: Amygdalin, Arbutin, Lactose, Mannitol, Melibiose, N-acetylglucosamine, Raffinose, Glucose and Sorbitol. One gram of sugar was added to one of the ten 100ml aliquot of the basal medium. The remaining bottle containing basal medium without carbohydrate was used as a negative control to show that the streptococci tested were unable to ferment the basal medium in the absence of carbohydrate. The sugars were autoclaved at 115°C for 15 minutes and stored at 4°C.

Aesculin Hydrolysis Test

The tryptone and yeast extract were purchased from Oxoid.

1g	Tryptone
0.5g	Yeast extract
1g	Sodium acetate ($C_2H_3NaO_2$)
0.05g	Ferric ammonium citrate ($C_6H_8O_7$)
0.5g	Aesculin
0.1ml	Tween 80 (Polyethylene glycol sorbitan monooleate)
0.5ml	Salt A
0.5ml	Salt B
100ml	DD-H_2O

The solution was autoclaved at 115°C for 15 minutes and stored at 4°C.

Arginine Hydrolysis Test

The peptone and yeast extract were purchased from Oxoid.

0.5g	Peptone
0.3g	Yeast extract
0.3g	Glucose
1g	Sodium acetate ($C_2H_3NaO_2$)
0.3g	L-Arginine
0.1ml	Tween 80 (Polyethylene glycol sorbitan monooleate)
0.5ml	Salt A
0.5ml	Salt B
100ml	DD-H_2O

The solution was autoclaved at 115°C for 15 minutes and stored at 4°C.

Salt A

0.16g	Calcium carbonate ($CaCO_3$)
0.16g	Magnesium sulphate ($MgSO_4$)
400ml	DD-H_2O

After autoclaving (121°C, 15 minutes), this solution was stored at 4°C for up to one year and used when required.

Salt B

0.8g	dibasic Potassium phosphate (K_2HPO_4)
8.0g	Sodium bicarbonate ($NaHCO_3$)
1.6g	Sodium chloride (NaCl)
0.8g	monobasic Potassium phosphate (anhydrous, KH_2PO_4)
400ml	DD-H_2O

After autoclaving (121°C, 15 minutes), this solution was stored at 4°C for up to one year and used when required.

The carbohydrate fermentation and aesculin and arginine hydrolysis tests were performed in sterile flat-bottomed microtitre trays fitted with individual lids (Sterilin). A colour change from purple to yellow indicated carbohydrate fermentation. A colour change from light brown to black indicated aesculin hydrolysis, while a colour change from yellow to orange after the addition of 45µl Nessler's reagent indicated arginine hydrolysis.

Enzyme Tests

The following 4-methylumbelliferyl-linked glycosides were used to test for the production of glycosidic enzymes: β-D-fucoside, β-N-acetylgalactosaminide, α-neuraminate, α-L-fucoside, β-N-acetylglucosaminide, α-glucoside, β-glucoside, α-galactoside, α-arabinoside and β-galactoside.

0.001g	4-methylumbelliferyl-linked glycoside
0.5ml	DMSO (dimethylsulphoxide)

The glycoside and DMSO were dissolved in a universal container (Sarstedt) and 9.5ml TES buffer was added to make a final volume of 10ml. The final working concentration of glycoside was 100μg/ml. The glycoside solutions were stored at -20°C.

The enzyme hydrolysis tests were performed in non-sterile flat-bottomed microtitre trays (Sterilin) and incubated for 4 hours at 37°C. The results were read under a UV light.

Tables 2.3 and 2.4 show the scheme used to identify the streptococci to species level. The identification scheme used in this study is discussed further by Beighton (Beighton *et al*, 1991).

2.2.4 16S rRNA Gene Sequence Identification

The oligonucleotide primers (Sigma-Genosys, Pampisford, Cambridgeshire, UK) used were:

27F	5'-AGAGTTTGATCMTGGCTCAG-3'
1492R	5'-TACGGYTACCTTGTTACGACTT-3'

PCR was performed in 0.5ml eppendorf tubes (ABgene, Epsom, Surrey, UK) in a thermocycler (MWG Biotech (UK) Ltd, Milton Keynes, Bedfordshire, UK). Two microlitres of the DNA template was added to a reaction mixture (50µl final volume containing 10X PCR buffer, 1.5mM $MgCl_2$, 1.25U of *Taq* DNA polymerase (Promega, Southampton, Hampshire, UK), 20pmol of each primer and 20µM of deoxynucleoside triphosphates (dNTPs) (Roche Diagnostics Ltd, Lewes, East Sussex, UK)). The PCR mixtures were denatured for 5 minutes at 94°C and then subjected to 30 cycles of amplification (1 minute of annealing at 94°C, 1 minute of elongation at 54°C and 2.5 minutes of elongation at 72°C). Finally, the samples were denatured at 72°C for 5 minutes. The samples were cleaned-up using the Qiagen QIAquick® kit (Qiagen Ltd, Crawley, West Sussex, UK) and the concentration of the amplified products was determined by reading the OD at 260nm using a spectrophotometer (Pharmacia Biotech, Cambridge, UK). The PCR products were amplified using the polymerase chain reaction, but using only a single primer. Depending on the concentration of the PCR product, the DNA was either used diluted or concentrated. Highly concentrated PCR products were diluted with DD-H_2O to make a volume of

4µl. Bigdye™ terminator cycle sequencing ready reaction solution (ABI PRISM Applied Biosystems, Warrington, Cheshire, UK) was diluted 1:4 with 5X reaction buffer. Two µl of the diluted ABI was added to 1µl (5pmol) of either the forward or reverse primer and 4µl PCR product (neat or diluted) in a 0.5ml eppendorf tube. The PCR mixtures were subjected to 99 cycles:

95°C	Rapid thermal ramp
95°C	for 10 seconds
50°C	Rapid thermal ramp
50°C	for 5 seconds
60°C	Rapid thermal ramp
60°C	for 4 minutes
4°C	Rapid thermal ramp
4°C	hold indefinitely

The temperature ramp was set to 1°C s^{-1}.

Before sequencing the PCR products, the DNA was cleaned up using cold centrifugation. In a 0.5ml eppendorf tube, 13µl of DD-H_2O was added to 7µl of the PCR product and kept on ice. Two µl 3M sodium acetate and 50µl 95% ice cold ethanol was added to the mixture and incubated on ice for 10 minutes. After cold centrifugation (4°C) at 14k for 20 minutes, the supernatant was removed and ice cold 70% ethanol added. Cold centrifugation was repeated for 15 minutes and the ethanol removed. The DNA at the bottom of the tube was dried for a few seconds at 95°C, resuspended in 20µl Template Suppression Reagent (TSR)

(ABI PRISM Applied Biosystems) and vortexed. The resuspended DNA was heated to 95°C for 2 minutes, vortexed and immediately placed on ice. The PCR products were sequenced using an ABI310 Genetic Analyser (PE Biosystems, Warrington, Cheshire, UK) and analysed using the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/) and BLAST at the National Centre for Biotechnological Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST).

2.3 Molecular Biology Techniques

The chemicals in this section were purchased from Sigma-Aldrich.

2.3.1 Gel Loading Buffer (FBX)(6X)

0.025 g	Bromophenol Blue
0.025 g	Xylene Cyanole
1.5 g	Ficoll 400

Distilled water was added to make the solution to 10 ml. The loading dye was stored at room temperature indefinitely.

2.3.2 0.5M EDTA (pH8.0)

186.1 g	EDTA (disodium salt)
800 ml	DD-H_2O

The solution was stirred vigorously and the pH adjusted with concentrated NaOH in order to dissolve the EDTA salt. Once a pH of 8 had been achieved by adding 0.1M or 1M NaOH or HCl, the volume was adjusted to 1 litre by adding more distilled water and autoclaved to sterilise (121°C, 15 minutes). This solution was stored at room temperature.

2.3.3 25X TAE Running Buffer

121.0g	Tris base
28.55ml	glacial acetic acid
50ml	0.5 M EDTA (pH8.0)
600ml	DD-H_2O

The solution was made up to 1000 ml by adding distilled water. The Running buffer was not autoclaved because it contained acetic acid. This solution was stored at room temperature.

2.3.4 1X TAE Running Buffer

40ml	25X TAE Running Buffer
960ml	DD-H_2O

This Running buffer was used as an electrophoresis buffer and to make agarose gels. The Running buffer was stored at room temperature.

2.3.5 Agarose Gels

All DNA samples (genomic and PCR products) were run on a 1% agarose gel made with 1X TBAE.

2.3.6 5X Sequencing Reaction Buffer

0.203g	Tris-HCl
4.85g	Magnesium chloride ($MgCl_2$)
80ml	DD-H_2O

The pH was adjusted to pH9.0 with 10M NaOH and brought up to 100ml with distilled water. The 5X Sequencing Reaction Buffer was stored at room temperature.

2.3.7 3M Sodium Acetate (pH5.5)

18.46 g Sodium acetate ($C_2H_3NaO_2$)

The pH was adjusted to pH5.5 with glacial acetic acid and the volume made up to 75 ml by adding water. The solution was sterilised by autoclaving and stored at room temperature.

2.3.8 Genomic DNA Isolation

Genomic DNA was extracted using Puregene™ DNA Isolation kit (Gentra Systems, Minneapolis, USA).

Antibiotic	Solvent/Diluent	Storage of solution			Storage of powder	Notes
		+4°C	-20°C	-70°C		
Penicillin (Benzyl) (Potassium)	Water		1 month	1 month	+4°C; protect from light and moisture	
Ampicillin (trihydrate)	Phosphate buffer, pH8.0, 0.1M	1 week	Unstable	1 month	+4°C; protect from light and moisture	
Erythromycin	95% Ethanol	1 week			+4°C stable 3 years; protect from light and moisture	
Vancomycin	Water	1 week	3 months		+4°C; protect from light and moisture	
Tetracycline (hydrochloride)	Water		NR[a]	NR[a]	Unopened vials: 2 years at room temperature	Made fresh on the day of use – tetracycline precipitates if frozen
Metronidazole	Water	1 week			+4-25°C; protect from light	Warming the solution in a waterbath (37°C) will help dissolve the metronidazole powder
Kanamycin	Water				+4°C; protect from light	
Gentamicin	Water				+4-25°C; protect from light and moisture	

Table 2.1: Preparation and Storage of Antibiotic Solutions

[a] Not recommended

	Gram Stain	Oxidase	Catalase	Growth in air	Growth anaerobically
Rothia species	GPR	-	+	+	-
Staphylococcus species	GPC	-	+	+	+
Streptococcus species	GPC	-	-	+	+
Neisseria species	GNC	+	+	+	-
Pseudomonas species	GNR	+	-	+	-

Table 2.2 Tests used to Identify the Genus of the Mercury-resistant Organisms isolated in the Cross-sectional and Longitudinal Studies

	1	2	3	4	5	6	7	8	9	10
S. mutans	-	-	-	-	-	100%	92%	86%	-	-
S. sobrinus	-	-	-	-	-	100%	6%	-	-	-
S. sanguinis	73%	-	-	-	33%	-	58%	58%	-	33%
S. parasanguinis	18%	100%	-	27%	100%	100%	27%	91%	18%	100%
S. gordonii	-	78%	-	100%	100%	22%	100%	11%	-	44%
Tufted fibril group	-	100%	-	100%	100%	-	-	-	-	25%
S. oralis	-	100%	100%	-	100%	100%	-	36%	-	64%
S. mitis	-	-	25%	-	-	100%	-	80% +	-	40%
S. vestibularis	-	-	-	-	-	66%	-	-	100%	100%
S. salivarius	71%	-	-	-	-	36%	64%	14%	93%	86%
S. intermedius	100%	100%	100%	-	100%	100%	100%	-	-	-
S. anginosus	-	-	-	-	-	100%	100%	-	-	100%
S. constellatus	-	-	-	-	-	19%	4%	-	-	2%

Table 2.4: Scheme for Glycosidic Enzyme activities of Viridans Streptococci
% = Percentage of strains that give a positive reaction for each test

Enzyme: 1, β-D-fucosidase, 2, β-N-acetylgalactosaminidase, 3, a-neuraminidase (sialidase), 4, α-L-fucosidase, 5, β-N-acetylglucosaminidase, 6, α-glucosidase, 7, β-glucosidase, 8, α-galactosidase, 9, β-galactosidase, 10, β-galactosidase

(Beighton *et al*, 1991)

	Amygdalin	Arbutin	Inulin	Lactose	Mannitol	Melibiose	N-Acetylglucosamine	Raffinose	Sorbitol	Aesculin	Arginine
S. mutans	70%	100%	100%	100%	100%	50-90%	100%	100%	100%	100%	-
S. sobrinus	-	-	84%	87%	4%	-	3%	16%	-	-	-
S. sanguinis	42%	83%	33%	100%	-	75%	100%	75%	42%	75%	100%
S. parasanguinis	18%	45%	-	100%	-	82%	100%	82%	9%	36%	100%
S. gordonii	100%	100%	100%	100%	-	11%	100%	11%	-	100%	100%
Tufted fibril group	-	100%	-	75%	-	-	100%	-	-	-	75%
S. oralis	9%	9%	-	100%	-	55%	100%	55%	-	18%	-
S. mitis	-	-	40%	80%	--	100%	100%	100%	-	-	-
S. vestibularis	50%	33%	-	66%	-	-	50%	-	-	50%	-
S. salivarius	29%	93%	79%	93%	-	7%	29%	79%	-	93%	-
S. intermedius	78%	-	-	97%	3%	-	100%	5%	5%	-	-
S. anginosus	≥90%	100%	-	≥90%	17%	-	100%	50-69%	-	100%	100%
S. constellatus	29%	-	-	55%	4%	-	100%	4%	-	100%	100%

Table 2.3 Scheme for Carbohydrate Fermentation of Viridans Streptococci
% = Percentage of strains that give a positive reaction for each test

(Beighton *et al*, 1991)

Chapter Three

Effect of Medium Composition on the Susceptibility of Oral Streptococci to Mercuric Chloride

3.0 Effect of Medium Composition on the Susceptibility of Oral Streptococci to Mercuric Chloride

3.1 Introduction

The *in vitro* susceptibility breakpoint concentration of an antimicrobial is determined from knowing the *in vivo* pharmacodynamic (PD) and pharmacokinetic (PK) properties of the drug (MacGowen & Wise, 2001) (Equation 3.1)

$$\text{Breakpoint concentration} = \frac{C\max}{et} f \times s$$

C_{max} = maximum serum concentration following a stated dose at steady state (usually 1 hour post-dose)
e = factor by which the C_{max} should exceed the MIC
f = factor to allow for protein binding
t = factor to allow for the serum elimination half-life
s = shift (or reproducibility) factor

Equation 3.1: Determination of the Breakpoint Concentration

Pharmacokinetic studies determine the absorption, distribution and elimination of drugs (Craig, 1998). These factors, combined with the dosage regimen, determine the time course of drug concentrations in serum, which in turn determine the time course of drug concentrations in tissue and body fluids. With respect to antimicrobials, the time course of drug concentrations at the site of infection is of special interest. Pharmacodynamics is the relationship between serum concentration and the pharmacological and toxicological effects of drugs.

As discussed in Chapter 1, before the discovery of antibiotics, mercury was used as an antimicrobial to treat bacterial diseases caused by organisms such as *Treponema pallidum* (Syphilis) and *Mycobacterium leprae* (Leprosy). However, since then it has been found that mercury is also toxic to human tissues and probably poisoned more people with the disease than it cured. For this reason, the PD and PK of mercury are not known and therefore its susceptibility breakpoint concentration has not been determined. Unlike antibiotic sensitivity testing, where the minimum inhibitory concentration (MIC) of the antimicrobial against an organism can be determined using standardised methods described by quality assurance committees, mercury susceptibility testing cannot be carried out in this manner (Wheat, 2001). This has led to the use of a wide range of methods to determine whether organisms are 'resistant' to mercury. Many authors have employed agar dilution using various agars depending on the organisms tested. For example, studies determining mercury resistance in the faecal flora have used MacConkey agar (Summers *et al*, 1993), bile aesculin azide agar (Summers *et al*, 1993), Mueller-Hinton II agar (Österblad *et al*, 1995), 5% defibrinated horse blood and Antibiotic Sensitivity Medium II Agar (Edlund *et al*, 1996) and Luria agar (Wireman *et al*, 1997). Various other studies have employed nutrient agar (Khor & Jegathesan, 1983, Nakahara *et al*, 1977a, 1977b), modified *Actinomyces* defined medium (MADM) (Lyttle & Bowden, 1993b), Mitis Salivarius agar (Summers *et al*, 1993), tryptose-glucose-yeast (TGY) agar (Timoney, 1978) and cysteine-free agar (Rudrik *et al*, 1985). Depending on the agar used, these studies have also used various mercuric chloride ($HgCl_2$) concentrations as the breakpoint value, ranging from 7.4μM – 125μM (2μg/ml - 34μg/ml) (Nakahara *et al*, 1977a,

Khor and Jegathesan, 1983, Rudrik *et al*, 1985, Zscheck & Murray, 1990, Avila-Campos *et* al, 1991b, Summers *et al*, 1993, Österblad *et al*, 1995, Edlund *et al*, 1996, Sadhukhan *et al*, 1997, Wireman *et al*, 1997, Huang *et al*, 1999, Nascimento *et al*, 1999, Kholodii *et al*, 2000, Pike *et al*, 2002a, 2002b, Pike *et al*, 2003). Other studies have employed disc diffusion on a variety of agars (Dyke *et al*, 1970, Hall, 1970a, 1970b). In addition, the incubation conditions and times for which the plates were incubated varied according to the organisms tested.

3.2 Aims

As very little information was available on mercury sensitivity testing, the purpose of this study was to determine the most suitable agar and concentration of mercuric chloride to use in this project. This agar would be used to isolate the mercury-resistant organisms from the patients' samples and to determine the MIC of mercuric chloride for each organism, as described in Chapters 4 and 5.

3.3 Materials and Methods

3.3.1 Bacterial Strains

A total of 99 oral streptococci were isolated from the plaque and saliva of children attending a paediatric clinic at the Eastman Dental Hospital, London, UK. None of the children had received antibiotics during the three months prior to sampling. These organisms were isolated on Columbia agar (CBA) containing

5% defibrinated horse blood (E&O Laboratories, Bonnybridge, Falkirk, UK). The plates were incubated anaerobically at 37°C for 48 hours. The organisms were identified to genus level on the basis of their atmospheric requirements, morphology, Gram staining reaction, oxidase and catalase tests (Table 2.2). Streptococci were speciated using a sugar and enzyme identification system described by Beighton (Tables 2.3 and 2.4)(Beighton *et al*, 1991). Some streptococci were unidentifiable using biochemical methods and were identified using 16S rRNA sequencing (Lane, 1996).

Mercury-resistant strains of *Staph. aureus* NCTC 50581, *Ent. faecium* 664 1H1, *B. cereus* RC607 and *Enterococcus* CE13, as described in Chapter 2.1.7, were used as positive controls. Mercury-sensitive *Staph. aureus* 8325-4 and *S. mitis* 606 4T1 were used as negative controls.

3.3.2 Determination of the Minimum Inhibitory Concentration (MIC) of Mercuric Chloride

The MICs of $HgCl_2$ were determined on different media by the agar dilution method in 9cm plastic petri-dishes (Sarstedt, Leicester, UK). The following media were used: Columbia agar (CA); Columbia agar with 5% defibrinated horse blood; Tryptone Soya agar (TSA); Tryptone Soya agar with 5% defibrinated horse blood; Iso-Sensitest agar (ISOA); Iso-Sensitest agar with 5% defibrinated horse blood; Mueller-Hinton agar (MHA); Mueller-Hinton agar with 5% defibrinated horse blood; Brain Heart Infusion agar (BHIA); Brain Heart Infusion agar and 5% defibrinated horse blood; and Mitis Salivarius agar (MSA).

The 0.1M $HgCl_2$ stock solution was prepared by dissolving 0.272g mercuric chloride ($HgCl_2$) in 10ml sterile distilled water on the day of the test. This stock solution was stored in a plastic universal container (Sarstedt, Leicester, UK) wrapped in aluminium foil to protect from sunlight and was discarded if not used that day. The mercury agar plates were prepared on the same day that the MICs were determined. The agar was autoclaved in glass Duran bottles (Jencons) and allowed to cool to 50°C in a waterbath. The $HgCl_2$ stock solution was added to the agar to make plates with final concentrations between 1 and 2048μM (serial two-fold dilutions). Control plates without mercuric chloride were used to determine whether the various agars could support the growth of the control and test organisms. Before use, the agar plates were dried in a laminar flow cabinet for 20 minutes and kept out of direct light.

The test organisms were grown overnight on Columbia blood agar (CBA), harvested and emulsified in 3ml brain heart infusion (BHI) broth using a Q-tip. The broths were diluted with BHI to a 0.5 McFarland Standard ($5x10^6$ CFU/ml). The agar dilution test plates were inoculated with a multipoint inoculator (Mast, Bootle, Merseyside, UK), resulting in a final inoculum of approximately 10^4 CFU/spot (Steers *et al*, 1959). The plates were wrapped in aluminium foil and incubated in aerobic conditions at 37°C for 18 hours. The MIC was defined as the lowest concentration resulting in the absence of visible growth.

In order to assess the reproducibility of the MIC values obtained, the control organisms were tested up to 4 separate occasions.

3.4 Results

The MICs of $HgCl_2$ for the 99 oral streptococci and control strains are shown in Table 3.2, which highlights that the MICs vary depending on the agar used. For example, the MIC of $HgCl_2$ for the mercury-resistant *Staph. aureus* NCTC 50581 ranged from 32μM (MHA and TSA) to 1024μM (BHIA & blood and ISOA & blood). The table shows that the MICs obtained from Mueller-Hinton agar without blood were generally lower than the values obtained from the other 5 agars tested and the MICs obtained from Brain Heart Infusion agar with blood and Mitis Salivarius agar were generally higher. All MICs increased considerably when 5% defibrinated horse blood was added to the medium.

Table 3.2 shows the cumulative percentage of oral streptococcal isolates (99 strains) sensitive to mercury chloride in different growth media. Each point on the graph represents the total number of bacteria sensitive to a given concentration of mercuric chloride, including those which are sensitive to lower concentrations.

The streptococci were classified as resistant to $HgCl_2$ when the MIC value was equal to or greater than that of *Staph. aureus* NCTC 50581 when tested on the same agar. However, this organism was unable to grow on Mitis Salivarius agar, so the positive controls *B. cereus* RC607 and *Ent. faecium* 664 1H1 determine the breakpoint concentration on MSA. On Mueller-Hinton agar without blood only

39 out of the 99 streptococci (39.4%) tested were resistant to $HgCl_2$ (Table 3.4). However, when TSA without blood was used, 70.7% of the streptococci (70 out of 99) could be classified as being resistant.

Table 3.3 shows that the range of MIC values obtained varied according to the agar used. When Mitis Salivarius agar was used 9 different MICs were obtained, ranging from 2μM $HgCl_2$ to 512μM $HgCl_2$. With Mueller-Hinton (without blood) there were only 6 different MICs (4μM $HgCl_2$ to 64μM $HgCl_2$). Tryptone Soy (with blood), Iso-Sensitest (without blood), Iso-Sensitest (with blood), Columbia (without blood) and Columbia (without blood) also gave 6 MIC values.

Table 3.3 shows that for some agars the values obtained for the mercury-sensitive and mercury-resistant control strains were often identical. With the exception of TSA (with and without blood) all of the media tested were able to discriminate between the mercury-sensitive and mercury-resistant strains of *Staph. aureus*. However, in most cases the difference in MIC amounted to only one doubling dilution, although Columbia agar with blood showed two doubling dilutions difference between the sensitive and resistant controls.

Table 3.4 shows the MIC determinations of the mercury-sensitive and mercury-resistant control strains carried out on 4 separate occasions. *Bacillus cereus* strain RC607 was tested only on two separate occasions. The MHA (without blood) gave the most consistent MIC results. For 3 of the control organisms (*Staph. aureus* 8325-4, *S. mitis* 606 4T1, *Staph. aureus* NCTC 50581), the MIC values obtained using this medium were identical on each of the

4 occasions they were determined. The MIC values obtained using Columbia agar (with blood) showed far greater variation and, for one of the organisms, ranged over 4 doubling dilutions on the different testing occasions. This suggests that using CBA gives non-reproducible results.

3.5 Discussion

The culture medium used in antibiotic sensitivity tests plays a very important role and can have a significant influence on the results. Not only must it provide a standard, reproducible nutritional environment for the optimal growth of the organism(s) being tested, it must do so without affecting the activity of the antibiotics against the test organisms. In disc diffusion, it must also provide a suitable gel so that the antibiotics can diffuse freely in a uniform and reproducible manner. The cations Ca^{2+} and Mg^{2+} also strongly influence the MICs of antibiotics such as the aminoglycosides and tetracyclines (Garrod & Waterworth, 1971, Reller *et al*, 1974, Post & Bridson, 1991, Andrews, 2001a). Adding supplements to agar can also result in interactions between the agar components and antibiotics. For example, 5% lysed horse blood is added to agar containing trimethoprim and sulphonamides to reduce the levels of thymidine which antagonises the action of both antibiotics (Garrod & Waterworth, 1971, Andrews, 2001a). In addition, adding 5% defibrinated blood to the agar can also extend the range of its growth supporting ability (Ericsson & Sherris, 1971). Adding sodium chloride to agar results in more effective detection of MRSA (French *et al*, 1987).

A number of medium constituents are also known to bind to, or precipitate, mercury compounds resulting in a decrease in mercury's antimicrobial activity. Such compounds include metal ions, glutathione, cysteine, proteins and lipids (Rudrik *et al*, 1985, Farrell *et al*, 1990, Delnomdedieu & Allis, 1993, Girault *et al*, 1996, Latinwo *et al*, 1998). Unfortunately, blood, one of the most frequently used medium constituents in medical microbiology, contains many of these substances. Many of these substances contain large amounts of the element sulphur and it is known that mercury binds to thiol groups (-SH) resulting in mercury chelation, leading to less 'available' mercury and significantly reducing its bactericidal activity. Studies of media supplemented with mercuric chloride in dialysis chambers showed that after 48 hours, blood agar still bound 96% of the available mercury, compared with 50% binding by modified *Actinomyces* Defined Medium (MADM). Table 3.1 shows that in this study, incorporating blood into the agar resulted in an increase in the MICs of $HgCl_2$ of all the oral streptococci and control strains tested. Avila-Campos observed an increase in the MIC of $HgCl_2$ to 52 strains of the *B. fragilis* group when tested on Brain Heart infusion agar (BHIA) and 5% blood in comparison to the values obtained from BHIA *per se* (Avila-Campos *et al*, 1991b). Studies by Rudrik have previously used a semi-synthetic anaerobic medium devoid of cysteine (Rudrik *et al*, 1985). This agar contains a minimal amount of sulphur-containing amino acids resulting in less mercury chelation and thus greater bactericidal activity. The group found that by adding 1mM cysteine hydrochloride increased the MIC of $HgCl_2$ by up to 17-fold. This made a previously sensitive organism appear resistant.

Table 3.3 shows that the MICs determined on Mueller-Hinton agar were usually lower than when determined on the other 5 agars tested. Mueller-Hinton is recommended by the National Committee for Clinical Laboratory Standards (NCCLS) in antibiotic susceptibility testing and is probably the most widely used medium internationally (Brown, 1994). However, various manufacturers produce Mueller-Hinton agar and variability in cations, thymine and thymidine from these manufacturers and batches are encountered (Reller *et al*, 1974). The agar contains starch to ensure that toxic factors produced during growth are absorbed and also contains peptides in the form of casein hydrosylate. This agar is relatively simple in comparison to the others tested, which may result in fewer interactions between the agar ingredients and mercuric chloride.

The MIC values obtained when using Tryptone Soya agar and Columbia agar were generally greater than those obtained from the Mueller-Hinton agar. Both agars contain peptones, which are necessary for optimum growth of chemo-organotrophic organisms. These agars also contain sodium chloride. Previous studies by Gupta have described how the presence of sodium chloride in agar results in a decrease in the MIC of silver against *E. coli* strains by (Gupta *et al*, 1998). Chloride acts by increasing membrane permeability, resulting in an increase in the toxicity of Ag^+ to bacteria. These studies found that low concentrations of chloride made the differences in MICs between sensitive and resistant strains larger, while high concentrations of chloride increased the sensitivity to silver ions (Ag^+). This has also been demonstrated with mercury (Brown, 1994).

The MICs obtained using Iso-Sensitest agar were greater than those found using Mueller-Hinton, Columbia and Tryptone Soya agar. Iso-Sensitest is recommended by the Working Party of the British Society for Antimicrobial Chemotherapy (BSAC) for antibiotic sensitivity testing in the UK and, unlike Mueller-Hinton, is produced by a single manufacturer (Oxoid). Iso-Sensitest is a closely defined medium with stabilised mineral content and allows growth of a wide range of organisms without supplementation. It contains a large number of constituents such as peptones, sodium chloride and starch. In addition, it contains various sulphurous compounds such as colbalt sulphate, cupric sulphate, zinc sulphate and ferrous sulphate, which may result in a high degree of mercury chelation. Iso-Sensitest agar also contains L-cysteine hydrochloride (a sulphur-containing amino acid), biotin (a B vitamin containing sulphur) and menadione (non-sulphur containing vitamin K). Avila-Campos showed that adding menadione to BHIA resulted in a slight increase in the MIC of $HgCl_2$ towards isolates from the *B. fragilis* group (Avila-Campos *et al*, 1991b).

The MICs obtained from BHIA were the highest among all the 5 agars tested. Brain heart infusion is a highly nutritious agar, suitable for the culture of fastidious organisms such as pneumococci, streptococci, meningococci and dental pathogens. This highly proteinacious agar contains large quantities of beef heart and calf brain infusion solids and proteose peptones, which may result in the components interfering with mercury availability, possibly through chelation.

The MICs obtained with MS agar were greater than those obtained using Mueller-Hinton, Iso-Sensitest, Columbia and Tryptone Soya agar and, in some

cases, greater than those found with BHIA. Mitis Salivarius agar is a selective agar that selects for the growth of viridans streptococci and enterococci. Mitis Salivarius agar contains compounds necessary for the growth of these organisms, such as trypticase peptone, yeast extract, glucose and sucrose, and compounds that inhibit the growth of non-streptococcal organisms, such as trypan blue, crystal violet and potassium tellurite. One molecule of trypan blue contains four sulphur atoms ($C_{34}H_{24}N_6O_{14}S_4Na_4$) suggesting that mercury is chelating with these sulphur atoms, resulting in a decrease in bactericidal activity)(Figure 3.2).

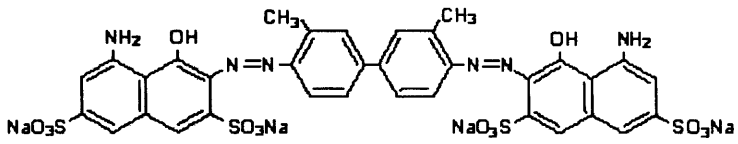

Figure 3.2: Trypan blue

Table 3.2 shows the results obtained from 99 oral streptococci that were able to grow on the MS agar. However, the table does not show the results of 30 oral streptococci that were unable to grow on MS agar (mercury-free control plates), but were able to grow on the other agars tested. Despite MS agar being selective for the isolation of oral streptococci and enterococci, previous studies have shown that MS agar gives lower counts of mutans streptococci than many non-selective media such as Brain Heart Infusion agar (Gold *et al*, 1973, Schaeken *et al*, 1986, Leistevuo *et al*, 2000). In addition, the *Staph. aureus* control strains were unable to grow on this agar, although MS agar was able to support the growth of the *B. cereus* RC607 mercury-resistant strain. Using this agar in the study would only select for mercury-resistant streptococci and not other genera. Although

streptococci account for a high proportion of organisms in the oral flora, 24% of the cultivable flora do not belong to this genus and belong to at least 30 other bacterial genera (Hardie, 1992, Chen *et al*, 1997).

In this study, the pH values of the agars used were very similar: BHIA, ISOA and MHA, were pH7.4, while TSA and CA were pH7.3 and MS was pH7.0. The pH of a medium also plays an important role in antibiotic sensitivity testing. For example, an acidic pH can result in an increase in the MIC of macrolides and therefore macrolide agar plates should be incubated in air and not carbon dioxide, unless the organism being tested is unable to grow in the absence of carbon dioxide. An acidic pH has been shown to increase the antimicrobial activity of mercury towards *Pseudomonas fluorescens* (Farrell *et al*, 1990). This group evaluated the toxicity of 3 different mercury salts; mercuric nitrate, mercuric chloride and mercuric acetate, in different pH conditions. They also studied the effect of chloride, citrate and cysteine on the toxicity of mercury. The work was carried out in liquid medium (M-IIY) and after incubation, growth was determined by measuring the optical density of the cultures. For each mercury salt, toxicity was greatest at pH6.0 and decreased significantly (P=0.05) at pH7.0. Increasing the pH to 8 had no effect on the toxicity of mercuric acetate or mercuric nitrate but significantly (P=0.05) reduced the toxicity of mercuric chloride. The toxicity of mercuric nitrate at pH8.0 was unaffected by the addition of citrate, enhanced by the addition of chloride and reduced by the addition of cysteine (a sulphur-containing amino acid).

In this study, the MIC of $HgCl_2$ on various solid media was compared. Previous studies have compared the antibacterial activity of mercury in broth and agar. Avila-Campos has demonstrated that the MIC_{50} and MIC_{90} in broth media were markedly lower than those determined using the agar dilution method, possibly due to better diffusion of the agent in broth medium (Avila-Campos *et al*, 1991b). In addition, solid medium contains agar, a complex mixture of polysaccharides extracted from red algae which contains sulphuric acid esters. The presence of these sulphuric compounds may also explain the increase in MICs of $HgCl_2$ on agar compared to broth.

It is essential that antimicrobial susceptibility tests provide reproducible results and the NCCLS suggest that with repeat testing, more than 95% of MICs should fall within an expected range, usually 3 $\log_2$ dilutions (Jorgensen, 1993). In this study, the 99 streptococci were not repeatedly tested, but both the positive and the negative control organisms were tested at least on 2 separate occasions. Some were tested on 4 separate occasions. Table 3.4 shows that when the mercury-sensitive and mercury-resistant controls were tested on 4 separate occasions, some of the results from some of the agars varied from test to test and were therefore not reproducible. While the majority of agars show values which range over only two doubling dilutions (BHIA, BHIA with blood, ISOA with blood, TSA with blood, MHA without blood, MHA with blood, CA without blood and MSA), ISO (without blood) and TSA (without blood) shows a range of 3 two-fold dilutions. Columbia agar (with blood) showed greater variation and, for *S. mitis* 606 4T1, ranged over 4 two-fold dilutions on the different testing occasions. Some agars showed far less variation. For example, for 2 of the

organisms, BHIA (with blood) and CA (without blood), the MICs obtained were identical on each of the 4 occasions they were determined. However, Mueller-Hinton agar (without blood) gave the most consistent results, where for 3 of the controls tested, the MIC values were identical on all of the testing occasions. For the other 2 organisms the MIC ranged over only 2 doubling dilutions.

In addition to giving reproducible MIC values, the medium must also be able to discriminate between mercury-sensitive and mercury-resistant control organisms and display a wide range of MIC values when used to test a group of organisms with different susceptibilities to mercury. Mitis Salivarius agar gave the greatest range of MIC values for the organisms tested (9), TSA (with blood), MHA (with blood), ISO (without), ISO (with blood), CA (with blood), CA (without blood), all gave the least range of MIC values for the organisms tested (6). All of the agars, except TSA (with and without blood) were able to discriminate between mercury-sensitive and mercury-resistant strains of *Staph. aureus* 8325-4. However, Mitis Salivarius agar was unable to support the growth of this organism. In addition, in most cases, aside from Columbia (without blood), the difference in MIC amounted to only one doubling dilution.

In conclusion, it appears that significant interactions between mercuric chloride, chelating agents and test media occur and that adding blood to solid media strongly decreases the antibacterial activity of mercury. The study highlighted that before undertaking studies of mercury resistance it is important to choose the correct agar. Not only must the agar support the growth of the organisms tested

but also its interaction with mercury must be determined, so that the correct concentration of mercury is utilised when determining mercury resistance. Looking at the results obtained in Tables 3.2 and 3.3, all of the agars tested have both advantages and disadvantages and it was difficult to easily choose a suitable agar. However, Mueller-Hinton appeared to be the most suitable as the MIC values of the control strains were very reproducible, it was able to discriminate between the mercury-sensitive and mercury-resistant control strains and displayed a wide range of MIC values for the randomly-picked group of oral streptococci tested. Furthermore, this agar was able to support the growth of all the streptococci tested, plus the control strains belonging to other genera. This agar is fairly nutritious and will support the growth of most micro-organisms found in the oral flora. In this study it was found that mercury-resistant control organisms *Staph. aureus* NCTC 50581, *Ent. faecium* 664 1H1 and *B. cereus* RC607 grow on agars containing 16μM, 32μM and 128μM mercuric chloride respectively (MIC 32μM, 64μM and 256μM). Based on these results, Mueller-Hinton agar containing 40μM $HgCl_2$ was used in the cross-sectional and longitudinal studies and the resistance to mercury of any mercury-resistant strains isolated on this agar were confirmed by determining the MIC of $HgCl_2$ for these organisms (Chapters 4 and 5). Österblad chose to use Mueller-Hinton agar containing 10μg/ml (37μM) $HgCl_2$ in their study (Österblad *et al*, 1995). In an environmental study, Henriette and co-workers used Mueller-Hinton agar containing 8μg/ml (29.6μM) $HgCl_2$ to screen Gram-negative organisms from an aerobic fixed-bed reactor (Henriette *et al*, 1991).

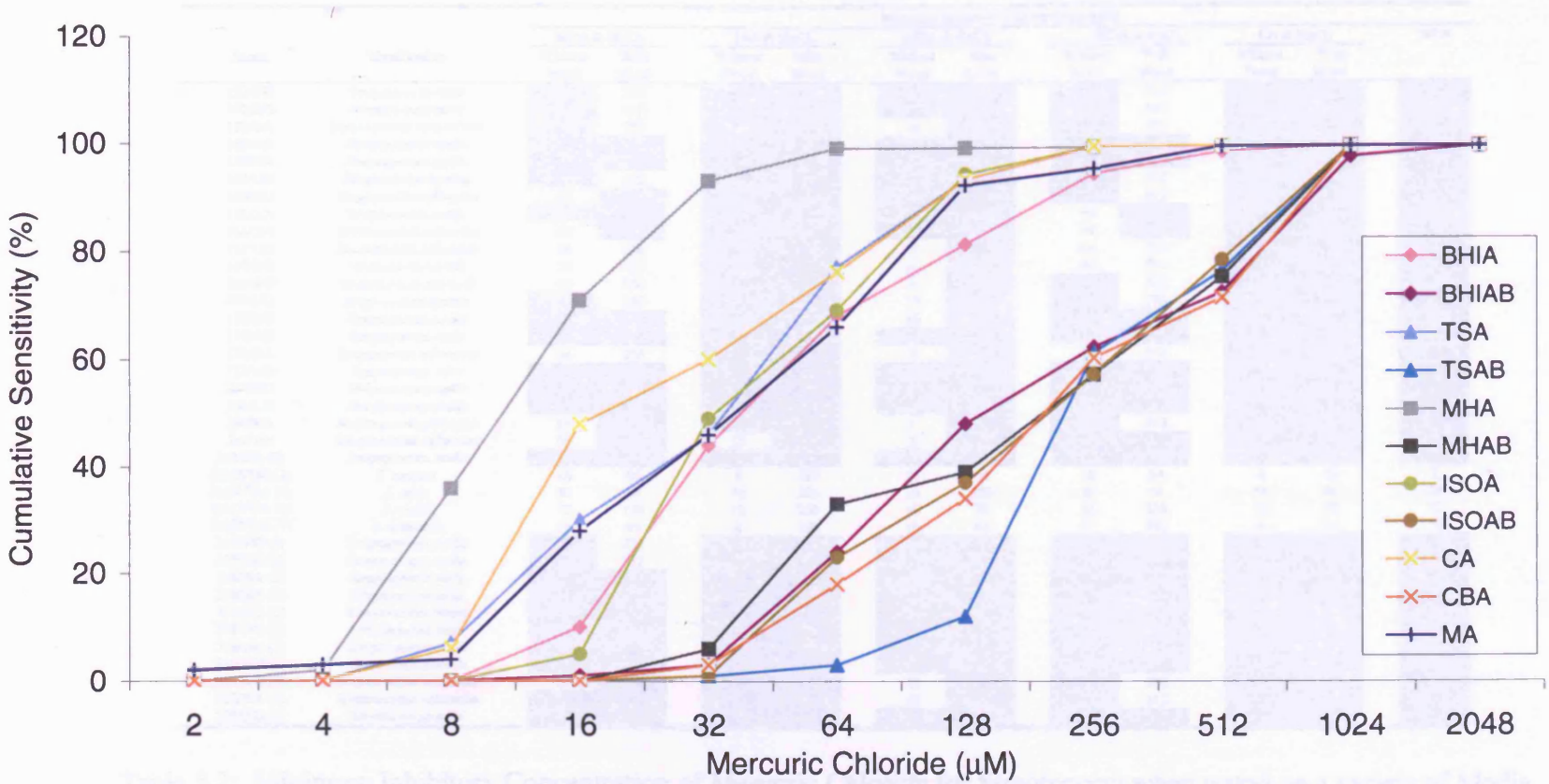

Figure 3.1: Cumulative percentage of Oral Streptococcal Isolates (99 strains) sensitive to Mercuric Chloride in different Growth Media

Strain	Identification	Minimum Inhibitory Concentration (μM)										
		BHIA & $HgCl_2$		TSA & $HgCl_2$		MHA & $HgCl_2$		ISOA & $HgCl_2$		CA & $HgCl_2$		MSA
		Without blood	With blood	Without blood	With blood	Without blood	With blood	Without blood	With blood	Without blood	With blood	
152/2/O	*Streptococcus mitis*	128	512	64	1024	32	512	128	512	64	1024	128
152/3/O	*Streptococcus mitis*	128	512	64	512	32	512	128	512	64	512	128
152/2/A	*Streptococcus constellatus*	128	256	64	512	16	512	128	512	64	512	128
159/1/A	*Streptococcus oralis*	256	1024	64	1024	32	1024	128	1024	128	1024	128
159/2/A	*Streptococcus oralis*	256	1024	64	1024	32	1024	128	1024	128	1024	128
160/1/O	*Streptococcus species*	256	512	128	512	32	512	128	512	128	1024	128
160/3/O	*Streptococcus salivarius*	64	1024	32	1024	32	512	128	512	64	512	128
162/1/A	*Streptococcus oralis*	256	1024	128	1024	32	1024	64	1024	128	1024	64
166/2/A	*Streptococcus salivarius*	64	1024	32	512	32	512	64	1024	64	512	128
167/1/O	*Streptococcus salivarius*	64	256	32	512	8	512	64	256	64	512	64
167/2/O	*Streptococcus oralis*	64	512	32	512	16	512	64	512	64	1024	128
169/4/O	*Streptococcus gordonii*	64	256	64	512	16	512	128	512	64	512	64
170/1/O	*Streptococcus species*	256	256	64	1024	16	512	128	256	128	1024	128
170/2/O	*Streptococcus oralis*	256	1024	64	1024	16	1024	128	1024	128	1024	128
170/4/O	*Streptococcus mitis*	256	1024	64	1024	32	1024	128	1024	128	1024	128
172/5/A	*Streptococcus salivarius*	64	256	32	512	8	512	64	512	64	512	64
173/1/O	*Streptococcus mitis*	256	1024	64	512	32	1024	128	1024	128	1024	128
204/4/O	*Streptococcus oralis*	256	1024	128	1024	64	1024	128	1024	128	1024	64
256/1/O	*Streptococcus oralis*	256	1024	64	1024	32	1024	128	1024	128	1024	128
264/4/A	*Streptococcus salivarius*	64	1024	32	512	8	1024	128	512	64	512	64
266/1/A	*Streptococcus salivarius*	64	1024	16	512	8	1024	256	1024	64	1024	128
313/2/O (3)	*Streptococcus oralis*	512	1024	256	1024	32	1024	256	1024	256	1024	512
321/3/T/O (1)	*S. sanguis*	16	64	8	128	16	64	16	64	8	64	2
321/1/T/A (1)	*S. mitis*	32	64	16	256	16	256	16	64	16	64	16
321/2/T/A (1)	*S. oralis*	32	128	16	256	16	64	32	256	16	128	32
321/3/T/A (1)	*S. sanguinis*	16	64	8	128	16	64	32	256	8	64	16
315/4/O (2)	*Streptococcus oralis*	128	512	64	1024	32	1024	128	1024	128	1024	128
315/5/O (2)	*Streptococcus oralis*	128	512	64	512	32	1024	128	1024	256	1024	64
316/2/A (2)	*Streptococcus mitis*	1024	2048	256	1024	32	1024	128	1024	256	1024	256
318/3/A (1)	*Streptococcus mitis*	512	2048	256	1024	64	1024	256	1024	256	1024	512
318/1/O (2)	*Streptococcus oralis*	512	1024	256	1024	64	1024	256	1024	256	1024	512
318/3/O (2)	*Streptococcus mitis*	512	1024	256	1024	64	1024	256	1024	256	1024	512
318/1/O (3)	*Streptococcus oralis*	128	1024	64	1024	32	1024	128	1024	128	1024	128
319/1/O (3)	*Streptococcus oralis*	128	1024	64	1024	32	1024	128	1024	128	1024	128
319/4/O (3)	*Streptococcus salivarius*	64	1024	32	512	16	1024	128	512	64	512	128
322/2/A (1)	*Streptococcus salivarius*	128	1024	32	512	16	1024	128	512	64	1024	128
325/2/O (1)	*Streptococcus mitis*	256	1024	128	1024	32	1024	128	1024	128	1024	256

Table 3.2: Minimum Inhibitory Concentration of Mercuric Chloride for Streptococci when tested on a variety of Media (Green shading indicates resistance)

Strain	Identification	Minimum Inhibitory Concentration (μM)										
		BHIA & $HgCl_2$		TSA & $HgCl_2$		MHA & $HgCl_2$		ISOA & $HgCl_2$		CA & $HgCl_2$		MSA
		Without blood	With blood	Without blood	With blood	Without blood	With blood	Without blood	With blood	Without blood	With blood	
325/3/O (1)	*Streptococcus mitis*	256	1024	128	1024	32	1024	64	1024	128	1024	256
325/2/A (1)	*Streptococcus species*	128	1024	64	1024	16	1024	128	512	128	1024	128
328/2/O (1)	*Streptococcus species*	64	128	128	256	16	256	64	256	32	256	64
328/3/O (1)	*Streptococcus species*	64	128	64	256	16	128	32	256	16	128	64
329/2/O (1)	*Streptococcus species*	32	128	64	256	8	64	32	128	16	128	32
329/3/O (1)	*Streptococcus species*	32	128	64	256	16	256	32	256	16	256	32
330/2/O (1)	*Streptococcus species*	64	512	128	256	16	512	64	256	16	256	64
330/3/O (1)	*Streptococcus species*	64	128	64	256	32	512	64	256	32	256	64
330/4/O (1)	*Streptococcus species*	64	128	64	256	8	128	64	128	32	64	64
330/2/A (1)	*Streptococcus species*	32	512	256	256	16	256	32	512	16	256	64
330/4/A (1)	*Streptococcus species*	64	128	128	256	64	512	64	128	32	256	128
331/3/O (1)	*Streptococcus species*	64	128	128	256	16	256	64	512	32	256	64
331/4/O (1)	*Streptococcus species*	64	128	128	256	16	128	64	512	32	256	128
331/2/A (1)	*Streptococcus species*	64	128	128	256	16	256	64	512	32	256	128
314/1/T/O (3)	*Streptococcus species*	256	1024	128	256	64	256	128	512	128	512	64
317/2/T/O (2)	*Streptococcus sanguinis*	16	32	8	128	4	64	32	64	8	64	16
317/1/T/A (2)	*Streptococcus mitis*	32	64	16	128	8	64	32	64	16	64	16
320/2/T/A (1)	*S.sanguinis*	16	32	8	128	4	32	16	64	8	32	2
321/2/T/O (1)	*S. gordonii*	16	16	8	32	16	32	16	32	16	64	16
321/2/T/O (2)	*Streptococcus species*	32	256	16	256	16	64	32	128	16	128	16
321/3/T/O (2)	*Streptococcus species*	32	256	16	256	16	64	32	128	16	128	16
317/2/T/A (2)	*S. parasanguis*	32	512	16	256	16	256	32	256	16	256	32
317/3/T/A (2)	*Streptococcus species*	16	64	16	256	8	64	32	64	8	256	16
317/1/T/O (3)	*Streptococcus oralis*	64	1024	32	1024	16	512	64	512	64	1024	128
317/2/T/O (3)	*S. salivarius*	32	128	128	256	8	256	32	256	16	256	32
317/2/T/A (3)	*Streptococcus oralis*	32	128	128	256	8	256	32	256	16	256	32
317/3/T/A (3)	*Streptococcus oralis*	128	1024	32	1024	16	512	64	512	64	1024	128
317/4/T/A (3)	*Streptococcus mitis*	32	64	64	256	8	64	32	64	16	64	32
318/1/T/O (1)	*Streptococcus species*	32	128	16	256	8	64	32	64	16	256	16
318/2/T/O (1)	*Streptococcus species*	32	128	16	256	8	64	32	64	16	256	16
318/3/T/O (1)	*Streptococcus species*	64	64	32	256	32	256	32	128	32	256	32
318/1/T/A (1)	*Streptococcus species*	32	64	16	128	8	32	16	64	16	64	16
318/3/T/O (2)	*Streptococcus species*	32	128	16	256	8	64	32	64	16	256	16
318/1/T/A (2)	*Streptococcus mitis*	32	128	16	256	8	256	32	128	16	256	16
318/2/T/A (2)	*Streptococcus mitis*	32	128	32	256	16	256	32	256	16	256	32
318/3/T/A (2)	*Streptococcus mitis*	32	64	16	256	8	64	32	128	16	128	16
318/4/T/A (2)	*S. parasanguis*	64	64	32	256	16	128	32	128	32	128	16
319/1/T/O (1)	*Streptococcus species*	64	64	32	256	8	128	32	128	32	256	32

Table 3.2 continued: Minimum Inhibitory Concentration of Mercuric Chloride for Streptococci when tested on a variety of Media (Green shading indicates resistance)

Strain	Identification	Minimum Inhibitory Concentration (μM)										
		BHIA & $HgCl_2$		TSA & $HgCl_2$		MHA & $HgCl_2$		ISOA & $HgCl_2$		CA & $HgCl_2$		MSA
		Without blood	With blood	Without blood	With blood	Without blood	With blood	Without blood	With blood	Without blood	With blood	
319/2/T/O (2)	*S. mitis*	32	64	16	256	8	64	32	128	16	128	16
319/4/T/O (2)	*S. mitis*	32	128	16	256	8	256	32	256	16	128	4
319/1/T/A (2)	*Streptococcus* species	32	256	16	256	8	64	32	64	16	128	16
319/2/T/A (2)	*Streptococcus* species	32	128	16	256	8	64	32	128	16	128	16
319/3/T/A (2)	*Streptococcus* species	32	128	16	256	8	64	32	128	16	128	16
320/1/T/O (1)	*S. sanguinis*	128	256	32	256	32	256	64	512	32	128	64
320/1/T/A (1)	*S. sanguinis*	64	256	16	256	8	512	64	512	16	128	64
321/1/T/A (2)	*S. mitis*	16	64	8	256	16	64	32	64	16	64	16
321/2/T/A (2)	*S. gordonii*	16	64	8	256	16	64	32	64	8	64	16
321/3/T/O (3)	*Streptococcus* species	128	256	32	512	16	128	64	256	64	512	64
321/1/T/A (3)	*Streptococcus* species	32	128	128	256	8	64	32	256	16	256	32
321/3/T/A (3)	*Streptococcus sanguis*	128	128	16	128	8	64	64	128	32	256	64
322/1/T/A (1)	*S. parasanguis*	64	256	16	256	16	512	32	256	16	128	64
322/3/T/A (3)	*S. parasanguis*	32	128	64	256	16	64	32	64	16	256	32
323/2/T/O (1)	*Streptococcus* species	32	256	16	256	16	256	32	256	16	128	16
325/1/T/O (2)	*Streptococcus gordonii*	16	64	64	64	8	64	32	64	16	32	8
325/2/T/O (2)	*Streptococcus* species	32	64	64	256	8	64	32	64	16	256	32
325/1/T/A (2)	*Streptococcus gordonii*	16	64	64	64	8	32	32	64	16	32	16
325/2/T/O (3)	*Streptococcus gordonii*	32	64	64	128	8	32	32	64	16	64	16
325/3/T/A (3)	*Streptococcus gordonii*	32	64	64	256	8	32	32	64	16	64	32
327/1/T/O (3)	*S. parasanguis*	32	256	64	256	16	256	32	256	16	256	32
327/3/T/O (3)	*Streptococcus mitis*	32	512	128	256	8	256	32	256	16	256	32
327/1/T/A (3)	*Streptococcus mitis*	32	64	64	256	8	64	32	64	16	64	32
327/2/T/A (3)	*S. parasanguis*	32	64	64	128	8	64	32	64	16	64	32
8325-4	*Staphylococcus aureus*	64	512	128	512	16	256	64	512	16	256	NG
606 4T1	*Streptococcus mitis*	32	64	16	256	8	64	32	64	16	256	16
NCTC 50581	*Staphylococcus aureus*	128	1024	32	512	32	512	128	1024	64	512	NG
664 1H1	*Enterococcus faecuim*	256	1024	128	512	32	512	128	1024	128	512	64
CE13	*Enterococcus* species	512	2058	128	1024	128	1024	256	1024	256	1024	512
RC607	*Bacillus cereus*	2058	2058	512	1024	128	1024	512	1024	256	1024	64
Number of resistant bacteria		31	27	70	38	28	42	30	21	40	40	53
% resistant bacteria		31.3	27.3	70.7	38.4	28.3	42.4	30.3	21.2	40.4	40.4	53.5

Table 3.2 continued: Minimum Inhibitory Concentration of Mercuric Chloride for Streptococci when tested on a variety of Media (Green shading indicates resistance)

Agar	MIC (μM)			Number (%) of Hg-resistant strains	MIC of positive control (μM)	MIC of negative control (μM)	Susceptibility of Hg-sensitive strain of *Staph. aureus*
	Range of MIC values (number of doubling dilutions)	MIC_{50}	MIC_{90}				
BHIA	16-2048 (8)	32	256	31 (31.3)	128	64	S
BHIA & blood	16-2048 (8)	256	1024	27 (27.3)	1024	512	S
TSA	8-512 (7)	64	128	70 (70.7)	32	32	R
TSA & blood	32-1024 (6)	256	1024	38 (38.4)	512	512	R
MHA	4-256 (7)	16	32	28 (28.3)	32	16	S
MHA & blood	32-1024 (6)	256	1024	42 (42.4)	512	256	S
ISOA	16-512 (6)	64	128	30 (30.3)	128	64	S
ISOA & blood	32-1024 (6)	256	1024	21 (21.2)	1024	512	S
CA	8-256 (6)	32	128	40 (40.4)	64	16	S
CA & blood	32-1024 (6)	256	1024	40 (40.4)	512	256	S
MSA	2-512 (9)	64	128	53 (53.5)	64	NG	NG

Table 3.3: Summary of MIC values for the Streptococcal Strains obtained using a range of Solid Media

NG = No Growth

Organism	Sensitivity to $HgCl_2$	MIC of $HgCl_2$ (μM)										
		BHIA		TSA		MHA		ISOA		CA		MSA
		Without blood	With blood	Without blood	With blood	Without blood	With blood	Without blood	With blood	Without blood	With blood	
Staph. aureus 8325-4	Sensitive	32 (3) 64 (1)	512 (4)	16 (2) 128 (2)	256 (2) 512 (2)	16 (4)	256 (3) 512 (1)	32 (2) 64 (2)	256 (2) 512 (2)	16 (4)	256 (4)	NG
S. mitis 606 4T1	Sensitive	16 (1) 32 (3)	64 (4)	8 (1) 16 (2) 64 (1)	128 (1) 256 (3)	8 (4)	32 (2) 64 (2)	16 (1) 32 (3)	64 (3) 128 (1)	16 (4)	32 (1) 64 (1) 128 (1) 256 (1)	16 (2) 32 (2)
Staph. aureus NCTC 50581	Resistant	64 (2) 128 (2)	512 (2) 1024 (2)	32 (3) 64 (1)	256 (1) 512 (3)	32 (4)	256 (2) 512 (2)	64 (1) 128 (2) 256 (1)	512 (2) 1024 (2)	32 (2) 64 (2)	256 (2) 512 (2)	NG
Ent. faecium 664 1H1	Resistant	128 (2) 256 (2)	512 (1) 1024 (3)	128 (3) 256 (1)	1024 (1) 512 (3)	64 (1) 32 (3)	512 (4)	128 (3) 256 (1)	512 (2) 1024 (2)	128 (4)	512 (3) 256 (1)	128 (1) 64 (3)
B. cereus RC607	Resistant	1024 (1) 2058 (1)	1024 (1) 2058 (1)	128 (1) 512 (1)	1024 (2)	128 (1) 256 (1)	1024 (2)	256 (1) 512 (1)	512 (1) 1024 (1)	128 (1) 256 (1)	1024 (2)	64 (2)

Table 3.4: Reproducibility of MIC of $HgCl_2$ for Mercury-sensitive and Mercury-resistant Control Strains
The values in brackets indicate the number of times this MIC value was obtained

NG = No Growth

Chapter Four

A Cross-sectional Study –

Resistance of the Commensal Oral Microflora to Mercury and Antibiotics in Subjects With and Without Dental Amalgam Fillings

4.0 A Cross-sectional Study – Resistance of the Commensal Oral Microflora to Mercury and Antibiotics in Subjects With and Without Dental Amalgam Fillings

4.1 Introduction

As discussed in Chapter 1, few studies have looked at the possibility that the release of mercury from dental amalgam fillings can act as selective pressure for mercury and antibiotic resistance in bacteria, especially those found in close proximity to the filling, such as the oral flora found in dental plaque. However, from the limited number of studies carried out, the results have been inconclusive. Summers' group found there to be a link between amalgam fillings and an increase in mercury and antibiotic resistance in the oral and faecal flora of primates (Summers *et al*, 1993). In contrast, Edlund found that, in human adults, there was no link between the presence of mercury amalgam fillings and the prevalence of mercury-resistant oral bacteria (Edlund *et al*, 1996).

4.2 Aims

There were three aims to this study. These were:

1. To determine whether children with fillings harboured a higher proportion of mercury-resistant bacteria in their oral flora than children without amalgam fillings.

2. The second aim was to identify the mercury-resistant bacteria and determine whether there were differences in the types of organism isolated from individuals with and without amalgam fillings.

3. The final aim was to investigate whether the mercury-resistant organisms were also resistant to 6 antibiotics and to determine whether there were differences between the non-amalgam and amalgam individuals. The MIC of mercuric chloride for these organisms was also determined during this part of the study.

4.3 Materials and Methods

4.3.1 Patient Selection

Healthy children, aged between 5-16 years, attending a clinic at the Department of Paediatrics at the Eastman Dental Hospital were enrolled in the study. Children with chronic medical disorders or those that had known viral carriage were not

recruited into the study. Children that had been treated with antibiotics during the proceeding 3 months were also excluded. During the visit to the clinic the dentist clinically assessed the patient and the child's guardian signed a consent form. The study was approved by the Ethics Committee of University College London.

The children were divided into two groups: Group 1 children had no amalgam fillings, while children in Group 2 had at least two tooth surfaces restored with amalgam.

4.3.2 Sample Collection

Plaque was collected from around the gingival margins and the surfaces of all the teeth using an alginate swab (MW&E, Medical Wire Equipment Co. (Bath) Ltd, Corsham, Wiltshire, UK). The swab was placed into a sterile glass bijou containing 4ml ¼ strength Ringers solution (Oxoid) and five glass beads (Sigma-Aldrich). One ml saliva samples were collected in mercury-free sterile plastic universal containers (Sarstedt).

4.3.3 Sample Processing

The fresh saliva and plaque samples were cultured within 30 minutes of arrival at the laboratory, where the swab from each patient was vortexed for 30 seconds, added to the saliva sample from the same patient and vortexed for a further 30 seconds. The sample mixtures were 10-fold serially diluted to 10^{-7} in Tryptone Soya broth (Oxoid).

Duplicate 100µl aliquots of dilutions (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) of each sample were inoculated onto mercury-free Mueller-Hinton agar. Duplicate 100µl aliquots of the undiluted sample and 10^{-1}, 10^{-2} and 10^{-3} dilutions were inoculated onto Mueller-Hinton agar containing 40µM $HgCl_2$. These plates were prepared on the same day that the specimen was collected and kept out of direct light before use. Four plates were used for each dilution, two of which were incubated aerobically and two anaerobically for 48 hours. During incubation, all the mercury-containing plates were wrapped in aluminium foil to protect from light.

Following incubation, colonies growing on the mercury-containing and mercury-free media were enumerated. Colonies growing on the mercury-containing agar showed little morphological differences and therefore at least four colonies from each patient (aerobic and anaerobic) were subcultured onto mercury-containing and mercury-free Mueller-Hinton plates. After 24-48 hours incubation (37°C, aerobic and anaerobic), the organisms were stored at -70°C, for identification and antibiotic-sensitivity testing at a later date.

4.3.4 Susceptibility Testing

Antimicrobial susceptibility was determined by an agar dilution technique, as described by the British Society for Antimicrobial Chemotherapy (BSAC)(Andrews, 2001b). Both the mercury and antibiotic plates were prepared on the day that they were inoculated. Mueller-Hinton agar was used to determine the MIC of $HgCl_2$, while Iso-Sensitest agar supplemented with 5% defibrinated

horse blood (E&O Laboratories) was used to determine the MICs of the antibiotics. The stock solutions used are described in chapter 2.1.4 and the range and breakpoints are shown in Table 4.1 below:

Antimicrobial	Range (mg/l)	Breakpoint[ab]	
		Gram-positive	Pseudomonads
Mercury	1-1024	16	16
Penicillin	0.008-8	0.125	N/A
Ampicillin	0.008-8	1	8
Erythromycin	0.008-8	0.5	N/A
Vancomycin	0.0625-16	4	N/A
Tetracycline	0.016-128	1	1
Gentamicin	0.03125-128	1[c] 128[d]	8

Table 4.1: Concentration Ranges and Breakpoints used in the Cross-sectional Study

[a] The mercuric chloride concentration was measured in μM. The antibiotic concentrations were measured in mg/l

[b] The breakpoint differs according to the organism tested. Penicillin is not used to treat pseudomonal infections, while all Gram-negative bacteria are inherently resistant to vancomycin and low-levels of erythromycin

[c] Breakpoint for all Gram-positive organisms except streptococci and enterococci. Streptococci and enterococci exhibit inherent low-level resistance to gentamicin

[d] Breakpoint for streptococci and enterococci

A Mueller-Hinton plate without mercuric chloride and an Iso-Sensitest plate without antibiotics were prepared to determine whether these agars could support the growth of the control and test organisms. Before use, the agar plates were dried in a laminar flow cabinet for 20 minutes and kept out of direct light.

The test organisms were grown overnight on Columbia blood agar (CBA), harvested and emulsified in 3ml brain heart infusion (BHI) broth using a Q-tip.

The broths were diluted with BHI to a 0.5 McFarland Standard ($5x10^6$ CFU/ml). The agar dilution test plates were inoculated with a multipoint inoculator (Mast) resulting in a final inoculum of approximately 10^4 CFU/spot. The plates were incubated in aerobic conditions at 37°C for 18 hours. The $HgCl_2$-containing plates were incubated in the same conditions, but wrapped in aluminium foil to prevent vapourisation of mercury. Organisms that were unable to grow under aerobic conditions were incubated in 5% CO_2 at 37°C for 18 hours. The MIC was defined as the lowest concentration resulting in the absence of visible growth.

4.3.5 Identification of Isolates

Oral isolates able to grow on the Mueller-Hinton agar containing 16μM $HgCl_2$ or greater were regarded as being mercury-resistant and were identified to the genus level using conventional microbiological techniques such as Gram stain, morphology, atmospheric requirements and standard biochemical tests (eg catalase and oxidase)(Table 2.2). The majority of the organisms were members of the *Streptococcus* genus and were further identified using an in-house biochemical technique or 16S rRNA sequencing (Tables 2.3 and 2.4) (Beighton *et al*, 1991, Lane, 1996).

The 16S rRNA gene was amplified using global primers (27F and 1492R) (Genosys-Sigma) via the polymerase chain reaction (PCR) and is described further in Chapter 2.2.4. The PCR products were sequenced using an ABI310 Genetic Analyser (PE Biosystems) and analysed using the Ribosomal

Database Project II and BLAST at the National Centre for Biotechnological Information (NCBI).

4.3.6 Statistical Analysis

The percentages of mercury-resistant bacteria from both the aerobic and anaerobic plates were analysed statistically. The mean, median and interquartile range (IQR) were determined to summarise the distribution pattern. The mean and IQR results from both incubation conditions and from both groups of patients were greater than the correlating median, suggesting that the results did not show normal distribution and were severely positively skewed (Figures 4.1 & 4.2). Therefore, the results could not be subjected to parametric testing. Initially, the non-parametric test used was the Mann-Whitney test using SPSS software (SPSS, Chicago, Illinois, USA). The Kolmogorov-Smirnov test was used to check that the overall shape and distribution of the data from each group did not differ significantly from each other.

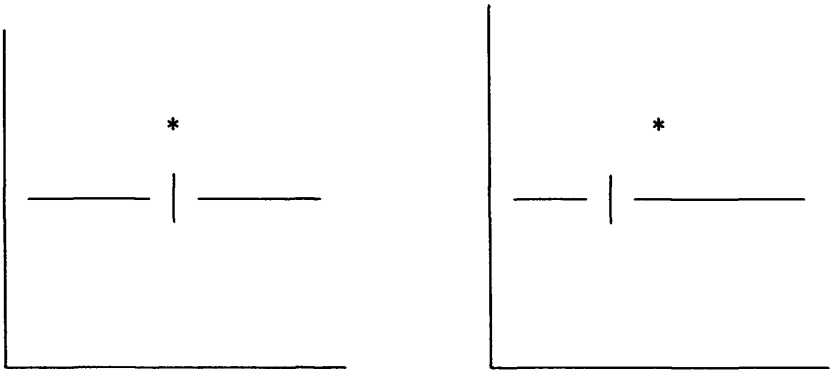

Figure 4.1: Dot chart represents normally (A) and non-normally (B) distributed data. The vertical line (|) represents the median and the asterisk (*) indicates the mean. The non-normally distributed data (non-parametric) is skewed to the right as the mean is greater than the median

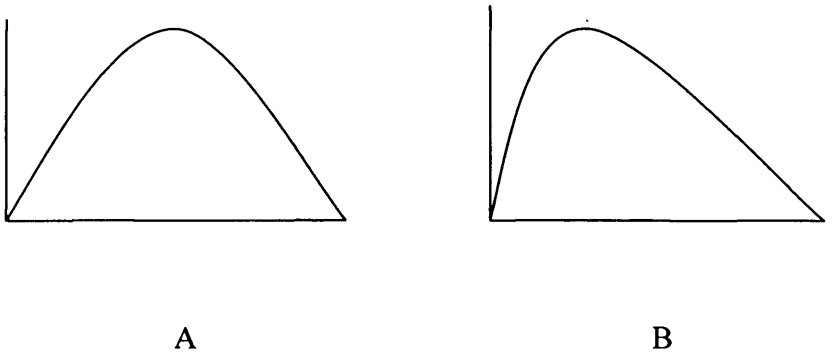

Figure 4.2: The bell-shaped curve shows normal (parametric) distribution (A), while the skewed curve shows non-normal (non-parametric) distribution (B)

Parametric tests are statistically more powerful than non-parametric tests, increasing the likelihood of correctly identifying a significant result. This can be achieved by transforming the data into a near-normal distribution by using a variety of mathematical options. In this study, adding a small offset to the data

and then transforming the results by the natural logarithm achieved this (Equation 4.1). The offset was added to allow zero values to be logged and also to maximise symmetry in both groups. The offset added to the aerobic data was 0.0008 and the offset added to the anaerobic data was 0.0011.

$$z = \ln(x+y)$$

y = offset value
x = original value
z = transformed value used in the parametric test

Equation 4.1: Data Transformation

The transformed data were analysed by multivariate multilevel regression using MLwiN software (Gilthorpe & Cunningham, 2000). All statistical tests were two-tailed, with the 5% level of statistical significance adopted throughout.

The proportion of mercury-resistant bacteria (as a percentage of both the aerobic and anaerobic viable counts) in the cultivable oral microflora of each subject was assessed in relation to the number of surfaces restored with amalgam using the Pearson (parametric) and Spearman's rho (non-parametric) correlation tests.

The Chi-square test was adopted to study whether there was a link between the presence of mercury and the identity of mercury-resistant organisms isolated from both groups.

4.4 Results

The 'amalgam' group consisted of 41 patients, mean age 10.7 years, SD 3.1; range, 6.3-16.8 years, with a mean of 4.7 amalgam surfaces (range 2-12). The control group ('no amalgam') consisted of 42 patients, mean age 10.2 years, SD 2.9, range 5.9-16.3 years. The sex of the children was not recorded.

Bacteria able to grow on Mueller-Hinton agar containing 16μM $HgCl_2$ and above were considered to be mercury-resistant, as the MIC of $HgCl_2$ for the two control mercury-resistant organisms, *Staph. aureus* NCTC 50581 and *Ent. faecium* 664 1H1, were 32μM and 64μM respectively. The mercury-sensitive organisms, *S. mitis* 606 4T1 and *Staph. aureus* 8325-4, were used as mercury-sensitive controls. These organisms were unable to grown on the agar containing 16μM $HgCl_2$.

In total, 139 mercury-resistant bacteria were isolated from 83 patients. Sixty four organisms were isolated from patients without amalgam and 75 bacteria were isolated from patients with amalgam.

Of those children who did not have amalgam fillings, 30 (71.4%) harboured mercury-resistant bacteria. A similar number (32) and proportion (78%) of children with amalgam fillings also harboured mercury-resistant bacteria.

Table 4.2 shows the proportions of mercury-resistant bacteria, expressed as percentages of the total viable aerobic and anaerobic counts, present in the

samples. The percentage range of organisms isolated from both sets of patients did not vary, either aerobically and anaerobically. The interquartile range (IQR) is a measure of the spread of the results. The mean and IQR for both incubation conditions and for both sets of patients were greater than the correlating median, suggesting that the results were not normally distributed and positively skewed (skewed to the right).

Under both aerobic and anaerobic conditions, it can be seen that the median numbers of mercury-resistant bacteria were greater amongst subjects with fillings than those without fillings. However, these differences between the filling and no filling group were not significant at the 5% level ($P \geq 0.05$) when using the non-parametric Mann-Whitney, whether analysing on the basis of the total viable aerobic count ($P=0.101$) or the total viable anaerobic count ($P=0.218$). The Kolmogorov-Smirnov test results were not significant ($P=0.225$ and $P=0.174$, aerobic and anaerobic respectively), concluding that the overall shape and distribution of the data from each group did not differ significantly from each other.

Table 4.2 shows that the transformed data analysed using multivariate multilevel regression were not significant, whether analysing on the basis of the total viable aerobic count ($P=0.107$) or the total viable anaerobic count ($P=0.256$).

Using the Pearson correlation test, analysis of the aerobic data from the amalgam-containing children revealed that the proportion of mercury-resistant bacteria (expressed as a percentage of the aerobic count) showed a significant

linear association with the number of surfaces present (r=0.45, P=0.004) (Table 4.3). However, the Pearson correlation coefficient test is sensitive to skewness and therefore this parametric test might not be reliable. Using Spearman's rank-correlation coefficient test, a non-parametric test, and the raw (non-transformed) data, the correlation and level of significance were largely reduced (ρ=0.23, P=0.151). In contrast, when the proportion of mercury-resistant bacteria was expressed as a percentage of the anaerobic count, only a borderline significant association with the number of amalgam surfaces present was found using either the Pearson (parametric) or Spearman's (non-parametric) correlation tests. The values calculated were r=0.31, P=0.053 and ρ=0.27, P=0.084, for the Pearson and Spearman's tests respectively.

There was no significant difference between the two groups in terms of the number of mercury-resistant species isolated, as shown in Table 4.4. Of the mercury-resistant bacteria isolated, 88% of those isolated from children with amalgam fillings were streptococci (Table 4.5). Similarly, 92% of the mercury-resistant organisms isolated from the children without amalgam fillings were identified as streptococci. Of those streptococci that could be identified, the majority were *S. oralis*. This was found in both patient types. However, mercury-resistant *S. oralis* was isolated more often from the amalgam group, and found to be statistically significant using the Chi square test ($\chi^2_{(1)}$=6.727, P=0.0095). In the non-amalgam group, the mercury-resistant isolates showed a greater species diversity with no particular streptococcal species being dominant in terms of frequency of isolation. Other mercury-resistant organisms isolated included *Staph. aureus* and coagulase-negative staphylococci

(CNS). A wider variety of bacterial species were isolated from patients with amalgam fillings. The bacteria isolated included *Rothia dentocariosa* and one Gram-negative organism; *Pseudomonas stutzeri*. No mercury-resistant obligate anaerobes were isolated from either group.

Once identified, the mercury-resistant bacteria were tested for their level of resistance to mercury, by determining the MIC of $HgCl_2$. The majority of organisms had an MIC of 32μM, although some had an MIC of 64μM (Table 4.6). One *S. mitis* strain (with amalgam) had an MIC of 128μM, while the *P. stutzeri* (with amalgam) had an MIC of 256μM.

Thirty-one (41%) and 21 (33%) of the mercury-resistant bacteria isolated from the groups with and without amalgam fillings respectively were also resistant to at least one of the antibiotics tested (Table 4.7). All of the isolates were facultative anaerobes and therefore metronidazole resistance was not tested. None of the mercury-resistant bacteria exhibited resistance to ampicillin or gentamicin. The mercury-resistant organisms were most often resistant to tetracycline (22%) and to a lesser extent, erythromycin (17%). Penicillin resistance was occasionally encountered in these organisms (6%). Ten isolates, six from patients with amalgam and 4 from patients without amalgam, were resistant to two antibiotics (Table 4.7). These isolates were either resistant to both tetracycline and erythromycin or tetracycline and penicillin. Tetracycline and erythromycin resistance was more common in organisms isolated from patients with amalgam fillings (5 organisms) than those without (1 organism). Additionally, in the amalgam group, four out of the five bacteria were identified as *S. oralis*.

Tetracycline and penicillin resistance was more common in mercury-resistant bacteria isolated from patients without amalgam fillings (3 organisms) than those with (1 organism). The *P. stutzeri* isolated from a patient with amalgam was resistant to three antibiotics – penicillin, vancomycin and erythromycin. However, members of the *Pseudomonas* genus are inherently resistant to these antibiotics, so this high resistance pattern was not significant.

As previously reported in this chapter, mercury-resistant *S. oralis* were more commonly isolated in this study than any other streptococcal species. In addition, 50% (18 out of 36) of the *S. oralis* isolated from children with amalgam were also resistant to at least one antibiotic. In contrast, only 24% (4 out of 17) of the *S. oralis* from the non-amalgam patients were resistant to an antibiotic. Statistically, using the chi square test, these differences were marginally not significant ($\chi^2_{(1)}$=3.32, P=0.068). Focussing on specific antibiotic resistance in the mercury-resistant *S. oralis* isolated, 12 out of 36 (33%) of the organisms isolated from patients with amalgam were resistant to tetracycline, while only 1 out of 17 (6%) *S. oralis* strains from the non-amalgam children were resistant to tetracycline. Statistically, using the chi square test, these results were significant ($\chi^2_{(1)}$=4.7, P=0.03). Similarly with erythromycin, 10 out of 36 (28%) *S. oralis* strains from the amalgam group were resistant, while only 2 out of 17 (12%) of the *S. oralis* isolates from patients without amalgam were resistant to erythromycin. However, these results were not significant using the chi square test ($\chi^2_{(1)}$=1.69, P=0.197).

Tables 4.9 and 4.10 show the MIC range, MIC_{50} and MIC_{90} of the 6 antibiotics tested and percentage of resistance for all the streptococci isolated. All isolates from the amalgam and non-amalgam groups were sensitive to ampicillin (MIC_{90} = 0.25 and 0.5mg/l). All isolates displayed vancomycin sensitivity with MIC_{50} and MIC_{90} values of 0.5 and 1mg/l for both the non-amalgam and amalgam groups. In the non-amalgam group the rank order of susceptibility for the viridans streptococci was: vancomycin = gentamicin = ampicillin (100%) > penicillin (94%) > erythromycin (85%) > tetracycline (82%). The same pattern was also observed with the amalgam group: vancomycin = gentamicin = ampicillin (100%) > penicillin (97%) > erythromycin (79%) > tetracycline (72%). For both the non-amalgam and amalgam patients, more organisms were resistant to tetracycline than any of the other antibiotics tested, with the highest MIC_{90} values (16 and 64mg/l) and 18 and 28% resistance proportions.

Tables 4.11 and 4.12 show the MIC range, MIC_{50} and MIC_{90} of the 6 antibiotics tested and percentage of resistant strains for *S. oralis*, *S. mitis* and *Streptococcus* species (including the unspeciated streptococci, *S. salivarius*, *S. parasanguinis*, *S. sanguinis*, *S. anginosus* and *S. vestibularis*). The MIC range of vancomycin for the streptococci was narrow, with MIC_{90}s of 0.5 and 1mg/l. All of the organisms tested were sensitive to vancomycin. In contrast, the MIC ranges of the other antibiotics, especially erythromycin and tetracycline were much larger. Due to the large MIC range for these antibiotics, there were considerable differences between the MIC_{50} and MIC_{90} values. The results for the coagulase-negative staphylococci, *Staph. aureus*, *R. dentocariosa* and *P. stutzeri*

are not listed in the table because of the limited number of these organisms isolated.

4.5 Discussion

The first aim of this study was to determine the number of children, with and without amalgam fillings, who harboured mercury-resistant bacteria in their oral flora and the prevalence of these organisms. Similar numbers of children without and with amalgam fillings harboured mercury-resistant bacteria in their oral flora (71.4% and 78% respectively). Summers reported that 63% of the human faecal samples tested in their study harboured mercury-resistant bacteria group (Summers *et al*, 1993). A similar study carried out by Ready and colleagues found similar proportions of mercury-resistant bacteria in saliva (Ready *et al*, 2003). Of 76 amalgam-free children tested, 54 (71%) harboured oral bacteria resistant to mercuric chloride despite using a different medium to the current study to isolate the mercury-resistant bacteria. The group used Mueller-Hinton agar containing 5% defibrinated horse blood plus 350μM mercuric chloride. As discussed in the previous chapter (Chapter 3.5), blood contains many constituents which can bind to mercury, resulting in less available mercury and significantly reducing its bactericidal activity.

In the current study, there were no statistically significant differences in terms of the proportions of mercury-resistant bacteria between the non-amalgam and amalgam groups. However, analysing the aerobic amalgam data using the Pearson correlation test revealed that the proportion of mercury-resistant bacteria

showed a significant linear association with the number of amalgam surfaces present. Several other investigators have shown that there are higher concentrations of mercury in the saliva and faeces of individuals with amalgam fillings than those without (Lyttle & Bowden, 1993b, Summers *et al*, 1993, Österblad *et al*, 1995, Edlund *et al*, 1996, Leistevuo *et al*, 2002). However, only one of these studies has reported a statistically significant linear trend between the number of amalgam-filled surfaces and the logarithm of total mercury in saliva (Leistevuo *et al*, 2002). This group found the average level of mercury in the (amalgam) group was 174nmol/l, 23 times higher than in the non-amalgam patients. Worryingly, 20.5% of the amalgam patients had more than 250nml/l mercury in their saliva, which exceeds the European Economic Community (EEC) limit of mercury in sewage. This study confirms that amalgam fillings are one of the most important sources of mercury exposure in human saliva in developed countries. However, the link between the proportion of mercury-resistant bacteria and number of amalgam fillings has been disputed by other studies. A study carried out by Diaz-Mejia looked at mercury and antibiotic resistance in the oral flora of Mexican and Cuban subjects (Diaz-Mejia *et al*, 2002). The Cuban patients possessed more fillings than the Mexican patients, but showed no relationship between the number of fillings and mercury and antibiotic resistance.

Unfortunately, there are few studies with which the results of the present study can be directly compared and, to complicate matters, there are many methodological differences between published studies. One major problem is that a standardised breakpoint concentration of mercury has not been established,

which has led to the use of a variety of concentrations, ranging from 7.4μM – 350μM (2mg/l – 946mg/l)(Nakahara *et al*, 1977a, Khor & Jegathesan, 1983, Rudrik *et al*, 1985, Zscheck & Murray, 1990, Avila-Campos *et* al, 1991b, Summers *et al*, 1993, Österblad *et al*, 1995, Edlund *et al*, 1996, Sadhukhan *et al*, 1997, Wireman *et* al, 1997, Huang *et al*, 1999, Nascimento *et al*, 1999, Kholodii *et al*, 2000, Pike *et al*, 2002a, 2002b, Pike *et al*, 2003, Ready *et al*, 2003). The agar used often depends on the type of bacteria tested. For example, MacConkey and bile aesculin azide agar has been used to determine mercury resistance in the faecal flora, while mitis salivarius agar has been used to isolate mercury-resistant oral streptococci (Summers *et al*, 1993). Other studies have used 5% defibrinated horse blood and Antibiotic Sensitivity Medium II Agar (Edlund *et al*, 1996), 5% defibrinated horse blood and Mueller-Hinton agar (Ready *et al*, 2003), Luria agar (Wireman *et al*, 1997), nutrient agar (Khor & Jegathesan, 1983, Nakahara *et al*, 1977a), modified *Actinomyces* defined medium (MADM) (Lyttle & Bowden, 1993b), tryptose-glucose-yeast (TGY) agar (Timoney *et al*, 1978) and cysteine-free agar (Rudrik *et al*, 1985). In addition, the conditions and times for which the plates were incubated varied according to the organisms tested.

To date, only four other studies have looked at the likelihood that mercury released from amalgam fillings could select for antibiotic- and mercury-resistant bacteria (Summers *et al*, 1993, Österblad *et al*, 1995, Edlund *et al*, 1996, Leistevuo *et al*, 2000). Of these, Summers studied antibiotic and mercury resistance in the faecal and oral flora of monkeys and Österblad looked at

resistance in the faecal flora of humans (Summers *et al*, 1993, Österblad *et al*, 1995). Edlund studied antibiotic and mercury resistance in the oral flora (and faecal flora) of humans, whereas Leistevuo looked at resistance in *S. mutans* isolated from human saliva (Edlund *et al*, 1996, Leistevuo *et al*, 2000). Summers' research differed to the others in that it was a longitudinal survey and will be discussed in Chapter 5 (Summers *et al*, 1993). The others were cross-sectional, investigating the flora isolated from patients without and with amalgam and, in two of the studies, from a group of patients that had their amalgam fillings removed and replaced with glass ionomers (Österblad *et al*, 1995, Edlund *et al*, 1996, Leistevuo *et al*, 2000).

Edlund found that mercury exposure from amalgam fillings was not a major factor in the selection of mercury and antimicrobial resistance in the oral and faecal flora (Edlund *et al*, 1996). Nevertheless, Edlund found a significant increase in the proportion of mercury-resistant *Bacteroides* species in the intestinal flora of adults with amalgam fillings compared to subjects without amalgam. However, they did not find significant differences in the proportion of mercury-resistant *E. coli* or enterococci from the two sets of patients. Unfortunately, several criticisms can be made regarding the experimental design of Edlund's study. Firstly, the group isolated mercury- and antibiotic-resistant organisms from only 20 adults (10 without and 10 with amalgam fillings), while the current survey studied resistance in organisms isolated from 83 children. Increasing the number of subjects in each group increases the reliability of the data analysis. Secondly, Edlund's study isolated mercury-resistant bacteria from only saliva samples and not saliva and plaque. The latter gives a more representative sample of the oral

microflora. Drucker and Jolly found that the percentage of patients with streptococci resistant to penicillin and erythromycin appeared to be greater when samples were obtained from the gingival margin than when saliva *per se* was sampled (Drucker & Jolly, 1971). Furthermore, an increase in the number of sites sampled would increase the likelihood of detecting antibiotic-resistant strains. Thirdly, Edlund's group included blood in the Antibiotic Sensitivity Medium II agar used to isolate the mercury-resistant bacteria, which as discussed in Chapter 3 binds to mercury (Avilo-Campos *et al*, 1991b, Lyttle & Bowden, 1993b, Leistevuo *et al*, 2000). The group incorporated 50μM $HgCl_2$ into their agar, which is a low concentration given that they also added 5% defibrinated horse blood. The authors do not report why they chose to use this concentration. All the patients in the study harboured mercury-resistant bacteria in their saliva, while the proportion (median) of mercury-resistant bacteria was around 50% in both sets of patients. This is in marked contrast with the present study where the proportions of mercury-resistant bacteria were less than 1% of the total viable count. However, Edlund's, group checked that these mercury-resistant organisms were not false positives by determining the MICs of $HgCl_2$ and using *E. coli* ATCC 25922, a mercury-sensitive organism (MIC 25μM) as a control. The group should have used a mercury-resistant control and any isolates with a MIC of $HgCl_2$ equal to or greater than that of the control, could be classified as mercury-resistant. These final points may suggest that many of the organisms classified as mercury-resistant in Edlund's study were not truly resistant.

In a study of the susceptibility of *S. mutans* to mercury and to 3 antibiotics, Leistevuo found that the MICs of the 4 antimicrobials did not differ for isolates from patients with amalgam, without amalgam and those who had amalgam removed (Leistevuo *et al*, 2000). The group determined the MICs of $HgCl_2$ on Mitis Salivarius agar using a series of concentrations ranging from 2-128mg/l (7.4-473.6μM). They found that the MICs of $HgCl_2$ for the 455 *S. mutans* tested were between 4-32mg/l (14.8-118.4μM). However, they did not state at which concentration the organisms were considered mercury-resistant or which, if any, organisms were used as mercury-resistant controls. In addition, Leistevuo did not mention the percentage of organisms from each group resistant to mercury, but report only the concentration range, MIC_{50} and MIC_{90} results. From these results one can only conclude that more organisms from the group who had had amalgam removed were less susceptible to mercury than the organisms from the other two groups, as the former had a higher MIC_{90}. Additionally, the group did not mention the breakpoint concentration for the 3 antibiotics tested or the percentage of organisms resistant to these antibiotics. Again, the concentration range, MIC_{50} and MIC_{90} were reported, which were very similar for each group, which may suggest that very few differences in antibiotic resistance were observed between the three groups.

Österblad grouped subjects on the basis of whether they had amalgam fillings, had recently had them removed or had never had amalgam fillings (Österblad *et al*, 1995). No significant differences in either mercury or antibiotic resistance in the aerobic Gram-negative faecal flora of these subjects were seen. However, the group reported that both ampicillin and nalidixic acid resistance

were higher in the flora isolated from the amalgam group than the other two groups. They also showed a strong correlation between multiple antibiotic resistance with mercury resistance; 36% of all mercury-resistant strains were also resistant to two or more antibiotics. Like the present study, this group used Mueller-Hinton agar and both studies used a similar concentration of $HgCl_2$ to isolate mercury-resistant bacteria. Österblad incorporated 10mg/l (37μM) $HgCl_2$, while the present study used 40μM $HgCl_2$. However, Österblad plated the diluted faecal samples directly onto MacConkey agar and after incubation, replica plated onto the mercury- and antibiotic-containing agar, whereas the present study inoculated diluted samples directly onto mercury-containing agar. In both studies, mercury resistance was confirmed by determining the MIC of $HgCl_2$ using agar dilution. Österblad only reported the percentage of subjects harbouring ≥1% mercury-resistant bacteria and found that 21%, 21% and 15% subjects without amalgam, removed amalgam and with amalgam respectively harboured mercury-resistant bacteria. In contrast, the present study reported the number of subjects who harboured ≥0.00014% mercury-resistant bacteria. If the present study, like Österblad, had reported the number of patients harbouring ≥1% mercury-resistant bacteria in their oral flora, 21.4% patients without amalgam and 22% patients with amalgam harboured mercury-resistant bacteria. Unfortunately, Österblad's group did not report how many fillings the subjects had or had removed. Previous studies have shown that the number of amalgam fillings is a significant variable in predicting amalgam mercury exposure in humans (Leistevuo *et al*, 2002). Additionally, the number, gender and ages of the subjects in the 3 groups were not well-matched. Österblad collected faecal samples from 92 subjects with amalgam fillings, but only enrolled 56 and 43

subjects in the removed amalgam and non-amalgam groups respectively. Three times more female subjects than male were in the non-amalgam and removed amalgam groups, while the mean age of the subjects in the non-amalgam group was 22, compared to 48 years of age in the amalgam group and 50 years of age in the removed amalgam group. Ideally, the groups studied should be well-matched with respect to age and gender, but in practice this is usually impossible.

The second aim of the current study was to identify the mercury-resistant bacteria and determine whether there was a difference in the type of organisms isolated from the two groups. The majority of mercury-resistant bacteria isolated from both groups were streptococci. Previous studies have shown that streptococci are the predominant cultivable oral flora in children between the ages of 2 and 10 years (Chen *et al*, 1997, Kamma *et al*, 2000). Furthermore, in the case of the amalgam group, a large proportion were *S. oralis*. *Streptococcus oralis* is a common member of the microflora of several oral habitats including supragingival plaque, saliva and mucosal surfaces. A study by Lucas found the predominant oral streptococci in children aged between 5 and 16 years to be *S. salivarius* (Lucas *et al*, 2000). Two other major species were *S. oralis* and *S. mitis*. In the present study *S. mitis* and *S. salivarius* were the second and third most commonly isolated streptococcal species. Similar work carried out by Ready *et al* (2003) also found that streptococci were the most commonly isolated mercury-resistant genera found within the oral flora. However, the group found that *S. mitis* were more predominant than *S. salivarius*, *S. sanguinis* and *S. oralis*, which were the second, third and fourth most commonly isolated streptococcal species.

The MIC of $HgCl_2$ was determined against all the mercury-resistant organisms isolated. The MICs ranged from 32-256μM, with MIC_{50} and MIC_{90} values of 32 and 64 respectively. The majority of streptococci had an MIC of 32 or 64μM, while only one *S. mitis* had a MIC of 128μM (with amalgam). Ready isolated many more streptococcal species with higher MICs than the present study (Ready *et al*, 2003). This group used the same methods to determine the MIC as the present study and isolated *S. sanguinis*, *S. mitis* and *S. oralis* with MICs of 128, 256 and 512μM respectively.

Few researchers have studied the susceptibility of oral bacteria to mercury and even fewer have studied mercury resistance in oral streptococci. Lyttle and Bowden tested the ability of oral streptococci and *Actinomyces* to grow in the presence of mercury (Lyttle & Bowden, 1993b). They found that streptococci were more resistant to mercury than *Actinomyces* and all of the streptococcal strains tested were able to grow on solid media containing 5mg/l (18.5μM) $HgCl_2$. They also found that *S. oralis*, *S. mitis* and *S. sanguinis* were able to adapt to growth in levels of mercury that would inhibit them on initial isolation.

The final aim of this study was to investigate whether the mercury-resistant organisms were also resistant to any of 6 antibiotics and to determine whether there were differences between the two groups.

For several decades the increase in antibiotic resistance of pathogenic organisms has been well documented, while the antibiotic resistance patterns of commensal

organisms have been investigated to a lesser extent. However, several *in vitro* susceptibility studies have recently reported antibiotic resistance in oral streptococci (Doern *et al*, 1996, Traub & Leonhard, 1997, Teng *et al*, 1998). These organisms are regarded as opportunistic pathogens and commonly the causative agent of infective endocarditis (Johnson *et al*, 2001). However, during the last 20 years, viridans streptococci bacteraemia in neutropaenic cancer patients has become more common (Oppenheim, 1998, Tunkel & Sepkowitz, 2002). Therefore, the majority of antibiotic resistance surveillance studies on viridans streptococci have investigated the susceptibilities of bacteria isolated from blood cultures from endocarditis patients and, more recently, from neutropaenic cancer patients (Carratalá *et al*, 1995, Endtz *et al*, 1997, Johnson *et al*, 2001). At present, very few epidemiological studies have been published which look at the antibiotic susceptibility patterns of the oral flora isolated directly from saliva and plaque samples from healthy human subjects. For this reason, the majority of the studies referred to in this discussion have looked at the antimicrobial susceptibility patterns of viridans streptococci isolated from the blood cultures of patients with bacteraemia. However, care must be taken when comparing results from clinical isolates (pathogens) and commensal organisms isolated from healthy patients. Ideally, blood cultures should be taken before antibiotic treatment, although in many circumstances antibiotics are administered before the blood culture is taken. This is especially true with immunocompromised patients, such as neutropaenic cancer patients who are often on prophylactic treatment against Gram-negative infections and *Pneumocystis carinii* and take antibiotics long-term, such as the fluoroquinolones and trimethoprim-sulfamethoxazole (co-trimoxazole) (Carratalá *et al*, 1995, Kennedy *et al*, 2001, Tunkel & Sepkowitz, 2002,

Westling *et al*, 2002). Traub and Leonhard found that viridans and β-haemolytic streptococci isolated from hospitalised patients were, in general, more resistant to 24 antibiotics than the streptococci isolated from pharyngeal swabs of healthy medical students (Traub and Leonhard, 1997). However, surprisingly, they also found that the isolates from the healthy students were more resistant to the macrolide antibiotics. In another study, Westling and colleagues investigated resistance to penicillin in viridans streptococci isolated from non-neutropaenic and neutropaenic patients (Westling *et al*, 2002). Sixty four out of 74 of the neutropaenic patients had been treated prophylactically with antibiotics and nine of the streptococci isolated from these patients were resistant to penicillin. None of the organisms isolated from the non-neutropaenic patients, who had not been treated prophylactically with antibiotics, were resistant to penicillin. Diekema also found that viridans streptococci isolated from the blood cultures of patients with cancer were more resistant to antibiotics than those organisms isolated from the blood cultures of patients without cancer (Diekema *et al*, 2001). Therefore, organisms isolated from septicaemic patients may be more resistant due to the prophylactic antibiotic treatment, resulting in selective pressure.

Another difficulty, when trying to compare this study with others, is that most other studies have used the NCCLS guidelines to test the susceptibility patterns of viridans streptococci (Bantar *et al*, 1996, Wisplinghoff *et al*, 1999, Poutanen *et al*, 1999). However, the present study used the British Society for Antimicrobial Chemotherapy (BSAC) guidelines. The NCCLS recommends Mueller-Hinton agar, while the BSAC advise the use of Iso-Sensitest agar. Different breakpoints have been endorsed by the two committees and in general,

the breakpoints recommended by the BSAC are lower than those recommended by the NCCLS (Brown, 1994). For example, the BSAC breakpoint for tetracycline is 1mg/l, while the NCCLS breakpoint is 4mg/l. In addition, the NCCLS describe organisms as either sensitive, intermediate resistant (low-level resistance) or resistant (high-level resistance), especially with regard to penicillin, whereas the BSAC describe organisms as either sensitive or resistant. In many studies discussed in this chapter, organisms showing low-level resistance are described as 'non-susceptible' and only organisms displaying high-level resistance are described as resistant.

Another complication when trying to compare the results from the present study with others is that many of the viridans streptococci, especially those belonging to the '*S. milleri*' group (*S. anginosus*, *S. constellatus* and *S. intermedius*), require carbon dioxide for growth. In general, previous studies have incubated plates in CO_2. This can decrease the pH of the medium, often resulting in a two-fold increase in the MIC of macrolide antibiotics (Carratalá *et al*, 1995, Endtz *et al*, 1997, Tuohy & Washington, 1997). In the present study, when determining the MIC, all organisms were initially tested in air and only those that were unable to grow in these conditions were re-tested in 5% carbon dioxide.

A further problem encountered when comparing the results of this study with others is that many other studies have not identified the streptococci to the species level. This is probably due to time constraints and more conceivably to the difficulty in identifying these organisms using biochemical methods. This is especially true with the mitis group (Kawamura *et al*, 1999).

The viridans streptococci were once believed to be uniformly susceptible to β-lactam antibiotics, macrolides and tetracyclines (Pfaller and Jones, 1997). However, many studies have recently shown that the susceptibility patterns can vary according to the species of the organism. In a US study, it was found that 58% of *S. mitis* strains were resistant to penicillin, while only 26% of *S. sanguinis* were resistant to the same antibiotic (Tuohy & Washington, 1997). In contrast, 100% of the '*S. milleri*' strains tested were susceptible to penicillin. In the UK, the Public Health Laboratory found that 13% of *S. oralis*, 14% of *S. sanguinis* and 5.5% of *S. gordonii* isolated from the blood cultures of patients with endocarditis were resistant to penicillin (Johnson *et al*, 2001). In contrast, all of the *S intermedius*, *S. constellatus, S. bovis* type I and *S. mutans* strains tested were susceptible to penicillin. These differences in resistance are also seen with the macrolide antibiotics and the tetracyclines. Wisplinghoff and colleagues found that 26% of *S. oralis* and 46% of *S. mitis* strains in their study were resistant to tetracycline (Wisplinghoff *et al*, 1999). In Taiwan, Teng found 50% of *S. mitis* were resistant to erythromycin, while 100% of *S. mutans* were susceptible to this antibiotic (Teng *et al*, 1998). Generally, antibiotic resistance in any member of the '*S. milleri*' group is rare (Jacobs and Stobberingh, 1996, Bantar *et al*, 1996). These findings are in keeping with those of other investigators who found higher rates of resistance in *S. mitis* than in other *Streptococcus* species (Renneberg *et al*, 1997, Gershon *et al*, 2002). This suggests that *S. mitis* should be considered an indicator organism of increasing antimicrobial resistance in oral streptococci as well as pneumococci (Renneberg *et al*, 1997). This is highlighted

in the present study where, alarmingly, 44% of the *S.mitis* isolated from the non-amalgam patients were resistant to tetracycline.

Penicillin is the preferred drug of choice for the treatment of infections caused by α-streptococci (viridans) and β-streptococci, especially in the treatment of endocarditis, where it is used in combination with gentamicin to create a synergistic effect (Johnson *et al*, 2001). However, unlike β-haemolytic streptococci, where resistance to β-lactams is uncommon, viridans group streptococci have demonstrated resistance to penicillin and other β-lactams (Pfaller and Jones, 1997, Ieven *et al*, 2000, Johnson *et al*, 2001). Penicillin-resistant strains of viridans group streptococci were first reported in 1963 in the oropharynx of children receiving continuous penicillin prophylaxis against rheumatic fever (Naiman and Barrow, 1963). Generally, viridans streptococci exhibit low-level resistance to penicillin (MIC 0.25-4mg/l), as observed in this study and many others (Endtz *et al*, 1997, Wisplinghoff *et al*, 1999, Seppala *et al*, 2003). In this study, high-level penicillin resistance (MIC≥8mg/l) was not encountered. Nevertheless, some authors have reported a trend toward high-level penicillin resistance in *S. mitis* (Venditti, *et al*, 1989, Endtz *et al*, 1997, Poutanen *et al*, 1999, Ioannidou *et al*, 2001). However, as in this study, other groups have failed to show this trend (Watanakunakorn & Pantelakis, 1993).

Many of the streptococci in this study exhibited low-level resistance to gentamicin (51% from the non-amalgam group, 75% from the amalgam group), which is inherent resistance and not significantly important. The MIC values for

gentamicin in the present study are in line with those found in previous studies (Carratalá *et al*, 1995, Jacobs & Stobberingh, 1996, Tuohy & Washington, 1997, Johnson *et al*, 2001). To date, high-level resistance to aminoglycosides been demonstrated in *S. mitis, Streptococcus uberis, Aeromonas viridans* and *Gemella morbillorum* (Potgieter *et al*, 1992, Kaufhold & Potgieter, 1993, Kobayashi *et al*, 2003). Most of these organisms also demonstrated high-level penicillin resistance (MIC 16-32mg/l).

Vancomycin resistance was not observed in any of the streptococci isolated. Resistance to vancomycin is rarely encountered in viridans streptococci (Venditti *et al*, 1989, Carratalá *et al*, 1995, Teng *et al*, 1998, Wisplinghoff *et al*, 1999, Luh *et al*, 2000, Diekema *et al*, 2001, Ioannidou *et al*, 2001, Kennedy *et al*, 2001, Gordon *et al*, 2002). Low-level resistance to vancomycin (MIC 16-32mg/l) was first reported in a *S. mitis* in Slovakia in 1996 (Krčméry *et al*, 1996a).

Many of the mercury-resistant isolates (6% non-amalgam, 9% amalgam) were also resistant to two or more antibiotics. In the present study, 3% of the mercury-resistant bacteria were resistant to penicillin and tetracycline, while 4% of the isolates were resistant to erythromycin and tetracycline. This association was also observed by Ready who found that 8% of mercury-resistant isolates were resistant to penicillin and tetracycline, while tetracycline and erythromycin resistance was more common and present in 11% of the isolates (Ready *et al*, 2003). In both studies, organisms resistant to both penicillin and erythromycin were not observed. However, organisms resistant to penicillin and

erythromycin and another antibiotic such as either tetracycline and/or vancomycin were isolated. In the present study, resistance to penicillin, erythromycin and vancomycin was observed in a Gram-negative species, while Ready isolated 3 strains of viridans streptococci resistant to penicillin, erythromycin and tetracycline (Ready *et al*, 2003).

As discussed, in the current study, mercury-resistant *S. oralis* was the most commonly isolated streptococcal species. *Streptococcus oralis* is very closely related to *S. pneumoniae* and both organisms share 99% sequence homology (Whatmore *et al*, 2002). *Streptococcus pneumoniae* is also closely related to *S. mitis* and there is evidence of the exchange of genetic material between the 3 species (Kawamura *et al*, 1995). Uptake of DNA from *S. mitis*, *S. oralis* or *S. gordonii* by *S. pneumoniae* followed by recombination into the chromosome can result in the creation of 'new genes'. This results in susceptible genes being replaced by mosaic penicillin binding proteins leading to penicillin resistance (Dowson *et al*, 1993). In addition, erythromycin resistance genes have been shown to transfer, via conjugation, from viridans group streptococci to *S. pneumoniae*, and vice versa (Luna *et al*, 1999).

As previously discussed, seven mercury-resistant staphylococci were isolated in this study. The organisms were identified using 16S rRNA sequencing. Of the organisms isolated, two were *Staph. aureus*, one *Staph. hominis*, one *Staph. warneri* and one *Staph. epidermidis*. Two of the organisms were unidentifiable using 16S rRNA and are therefore referred to as coagulase-negative staphylococci. Mercury resistance in *Staph. aureus* has been studied quite

extensively. The *merA* gene can be found chromosomally, but is often on plasmid pI258 (Witte *et al*, 1986, Laddaga *et al*, 1987). In this study, the staphylococci isolated were fairly susceptible to the antibiotics tested. Only *Staph. hominis* showed resistance to penicillin, while the *Staph. aureus* from an amalgam patient showed resistance to tetracycline. Ampicillin- and erythromycin resistance was not observed in any of the staphylococci. In addition, gentamicin resistance was not encountered in any of the mercury-resistant staphylococci isolated. However, other studies have shown that gentamicin resistance is commonly encountered in all *Staphylococcus* species, although it is more common in CNS than in *Staph. aureus* (de Neeling *et al*, 1998, Schmitz *et al*, 1999, Henwood *et al*, 2000). Vancomycin resistance was not observed in any of the staphylococci isolated in this study. Generally, vancomycin resistance is uncommon in staphylococci and was first reported in a coagulase-negative *Staphylococcus* in 1981, 7 years before the first vancomycin-resistant enterococci (VRE) was isolated (Cherubin *et al*, 1981, Srinivasan *et al*, 2002). Since then several reports have described low-level resistance (MIC 8-16mg/l) in *Staph. epidermidis* and *Staph. haemolyticus* (Schwalbe *et al*, 1987, Krčméry *et al*, 1996b). These strains were also resistant to methicillin. The first vancomycin-resistant *Staph. aureus* was isolated in 1996 in Japan (Hiramatsu *et al*, 1998).

In this study, six mercury-resistant *Rothia dentocariosa* strains were isolated. Five were isolated from patients with amalgam, while only one was isolated from a patient without amalgam. This organism, a member of the family *Micrococcacae*, is a normal inhabitant of the oral cavity (Salamon & Prag, 2001). It is present in human saliva and is most frequently isolated from supragingival

plaque. Though it appears to be an organism of low virulence, the recent increase in the number of reported cases of *R. dentocariosa* bacteraemia has established the pathogenic potential of this organism. The most frequent clinical presentations include septicaemia, endocarditis and pneumonia (Kong *et al*, 1998). However, very few authors have looked at the antimicrobial susceptibility patterns of this organism and the ones that have are ones in which the organism caused an infection (Kong et al, 1998, Salamon & Prag, 2001). In 3 cases of bacteraemia, the organisms were found to be susceptible to penicillin, ampicillin, erythromycin, vancomycin and gentamicin (Salamon & Prag, 2001). In a case of endocarditis, the organism was found to be susceptible to penicillin, amoxycillin and erythromycin, but resistant to gentamicin (Kong *et al*, 1998). Susceptibility to vancomycin was not tested. In the present study, one of the isolates from a patient with amalgam was found to show low-level resistance to penicillin (0.5mg/l).

Only one Gram-negative mercury-resistant organism was isolated during this study and this organism was more resistant to mercuric chloride than any of the other mercury-resistant organisms isolated from the cross-sectional study. The MIC of $HgCl_2$ was 256μM and the organism was identified as *P. stutzeri* using 16S rRNA sequencing. It was isolated from a patient with amalgam fillings. The organism was found to be resistant to penicillin, erythromycin and vancomycin. However, all Pseudomonads are inherently resistant to these antibiotics. *Pseudomonas stutzeri* is a saprophyte, usually found in the environment and rarely causes human disease (Yan *et al*, 2001). It is often found as a contaminant in bottled water, contact lens solutions and water cooling systems and many studies

have looked at biocide resistance in this organism rather than antibiotic resistance (Papapetropoulou *et al*, 1994, Tattawasart *et al*, 1999). This organism has been shown to develop resistance to non-oxidising water treatment bactericides and acquire resistance after exposure to gradually increasing concentrations of biocides (Brozel *et al*, 1993, Russell *et al*, 1998). *Pseudomonas stutzeri* has been shown to be more sensitive to biocides, including mercury, and antibiotics than *P. aeruginosa* (Tattawasart *et al*, 1999). However, other groups have found that strains of *P. stutzeri* contain plasmids that harbour mercury and silver resistance genes (Haefeli *et al*, 1984, Barbieri *et al*, 1989). *Pseduomonas stutzeri* is not regarded as a member of the normal oral flora of man. However, Ready also isolated a mercury-resistant *P. stutzeri.* Unfortunately, the MIC of $HgCl_2$ was not reported (Ready *et al*, 2003).

In conclusion, this study has shown that mercury and antibiotic resistance is widely distributed among the oral microflora of healthy children, although dental amalgam alone is not a key factor in promoting its spread. Many other factors and agents detrimental to bacteria may act as confounding variables in this study, selecting for resistance and masking the possible effect of mercury in amalgam fillings. These could be components of food or drugs. Traces of antibiotics in food, such as meat, milk, vegetables and fruit, might have some effect (Corpet, 1993). Traces of mercury are often found in fish and some studies have exclusively employed volunteers that had not eaten fish for one month prior to and during the study (Edlund *et al*, 1996).

One of the main findings of the study is that mercury-resistant strains of *S. oralis* were isolated significantly more frequently from children with amalgam fillings than from those without. In addition, 50% of the *S. oralis* isolated from children with amalgam were also resistant to at least one antibiotic, while only 24% of *S. oralis* from non-amalgam patients were resistant to an antibiotic. Despite this, these differences were narrowly not statistically significant. However, it was statistically significant that 33% *S. oralis* isolated from patients with amalgam were resistant to tetracycline, while only 6% *S. oralis* strains from the non-amalgam children were resistant to this antibiotic.

An additional important outcome of this study was the finding that the proportion of mercury-resistant oral bacteria showed a significant correlation with the number of amalgam surfaces in the children's mouth (Table 4.3).

The most important outcome of this study was that mercury- and antibiotic-resistant organisms are common in the oral flora of children with and without amalgam fillings. Resistance to these commonly used antimicrobial agents suggests the need for comprehensive surveillance programmes to monitor resistance in these and other commensal organisms (Bax *et al*, 2000).

	Proportion of aerobic count (%)		Proportion of anaerobic count (%)	
	without amalgam	with amalgam	without amalgam	with amalgam
Range	0-15.00	0-14.88	0-17.32	0-9.28
Mean	0.593	1.263	0.825	0.97
Median	0.0083	0.085	0.014	0.055
Inter-quartile range	0.2848	0.7978	0.3261	0.7948
Mann-Whitney *P* value	0.101		0.218	
(Kolmogorov-Smirnov P value)	*(0.225)*		*(0.174)*	
Multivariate regression *P* value[a]	0.107		0.256	

Table 4.2: Proportions of Mercury-resistant Microbes in the Oral Microflora of Children with and without Amalgam Fillings

[a] Multivariate regression in this instance uses multilevel modelling to analyse the two outcomes (with respect to the amalgam groups) simultaneously in order to optimise the statistical power

Number of amalgam fillings	Proportion of aerobic count (%)
2	0.024
2	0.56
2	0.037
2	0.23
2	3.02
2	1.48
2	0.0012
2	0.046
2	0.962
2	0.0845
2	0.0081
2	0.062
2	0.66
3	0.26
3	0.25
3	0.24
3	0.777
4	0.0047
4	0.05
4	0.046
4	1.93
5	0.5
5	0.94
5	0.0066
5	0.501
5	5
5	4.44
6	0.0015
6	0.00033
6	0.149
6	0.18
6	0.82
7	0.88
7	0.44
7	14.881
8	0.0025
8	0.016
8	0.364
11	5
12	0.0013
12	13.11

Table 4.3: Proportion of Mercury-resistant Microbes in the Oral Microflora of Children with Given numbers of Fillings

Number of mercury-resistant organisms	Number (%) of patients	
	Without Amalgam	With Amalgam
0	12 **(28.6)**	9 **(22.0)**
1	10 **(23.8)**	11 **(26.8)**
2	11 **(26.2)**	9 **(22.0)**
3	5 **(11.9)**	5 **(12.2)**
4	3 **(7.1)**	5 **(12.2)**
5	1 **(2.4)**	1 **(2.4)**
6	0 **(0.0)**	1 **(2.4)**

Table 4.4: Number of Patients with a given number of Mercury-resistant Species

Organism	No. (%) of isolates from children	
	without amalgam fillings	with amalgam fillings
Streptococcus oralis	17 **(27)**	36 **(48)**
Streptococcus mitis	9 **(14)**	7 **(9)**
Streptococcus salivarius	8 **(13)**	5 **(7)**
Streptococcus sanguis	1 **(2)**	3 **(4)**
Streptococcus parasanguis	6 **(9)**	2 **(3)**
Streptococcus vestibularis	1 **(2)**	0 **(0)**
Streptococcus anginosus	2 **(3)**	0 **(0)**
Unidentified streptococci	15 **(23)**	13 **(17)**
Staphylococcus aureus	1 **(2)**	1 **(1)**
Coagulase-negative staphylococci	3 **(5)**	2 **(3)**
Rothia dentocariosa	1 **(2)**	5 **(7)**
Pseudomonas stutzeri	0 **(0)**	1 **(1)**
Total	64	75

Table 4.5: Identity of Mercury-resistant Bacteria from the Patient Groups

	No. isolates							
	without amalgam				with amalgam			
	Mercury concentration (μM)							
Organism	32	64	128	256	32	64	128	256
Streptococcus oralis	**14**	**3**	0	0	**34**	**2**	0	0
Streptococcus mitis	**8**	**1**	0	0	**3**	**3**	**1**	0
Streptococcus salivarius	**8**	0	0	0	**5**	0	0	0
Streptococcus sanguis	**1**	0	0	0	**3**	0	0	0
Streptococcus parasanguis	**6**	0	0	0	**2**	0	0	0
Streptococcus vestibularis	**1**	0	0	0	-	-	-	-
Streptococcus anginosus	**2**	0	0	0	-	-	-	-
Unidentified streptococci	**13**	**2**	0	0	**11**	**2**	0	0
Staphylococcus aureus	**1**	0	0	0	**1**	0	0	0
Coagulase-negative staphylococci	**3**	0	0	0	**2**	0	0	0
Rothia dentocariosa	**1**	0	0	0	**5**	0	0	0
Pseudomonas stutzeri	-	-	-	-	0	0	0	**1**
Total	**58**	**6**	0	0	**66**	**7**	**1**	**1**

Table 4.6: Identity and Minimum Inhibitory Concentration ($HgCl_2$) of Mercury-resistant Isolates

Antibiotic	No. (%) of Hg-resistant isolates exhibiting resistance	
	without amalgam fillings	with amalgam fillings
Penicillin	5 **(8)**	4 **(5)**
Ampicillin	0 **(0)**	0 **(0)**
Erythromycin	9 **(14)**	15 **(20)**
Vancomycin	0 **(0)**	1 **(1)**
Tetracycline	11 **(17)**	19 **(25)**
Gentamicin[a]	0 **(0)**	0 **(0)**
At least one antibiotic	21 **(33)**	31 **(41)**
One antibiotic only	17 **(27)**	24 **(32)**
Two antibiotics	4 **(6)**	6 **(8)**
Three antibiotics	0 **(0)**	1 **(1)**

Table 4.7: Resistance to antibiotics of Mercury-resistant Bacterial Isolates from Children

[a] Streptococci have inherent resistance to aminoglycosides and low-level resistance to gentamicin is not significant (MIC ≥2– 128mg/l)
A MIC ≥128mg/l gentamicin is high-level resistance

Mercury-resistant isolate	Number of isolates displaying resistance[a]													
	Tetracycline		Erythromycin		Penicillin		Vancomycin		One antibiotic		Two antibiotics		Three antibiotics	
	-Am	+Am	-Am	+Am	-Am	+Am	-Am	+Am	-Am	+Am	-Am	+Am	-Am	+Am
S. oralis	**1**	**12**	**2**	**10**	**1**	0	0	0	**4**	**14**	0	**4**	0	0
S. mitis	**4**	**2**	0	**1**	0	**1**	0	0	**4**	**2**	0	**1**	0	0
S. salivarius	0	0	**2**	0	0	0	0	0	**2**	0	0	0	0	0
S. sanguis	0	0	0	**1**	0	0	0	0	0	**1**	0	0	0	0
S. parasanguis	**3**	0	**2**	0	**2**	0	0	0	**1**	1	**3**	0	0	0
Unidentified streptococci	**3**	**4**	**3**	**2**	**1**	**1**	0	0	**5**	**5**	**1**	**1**	0	0
S. aureus	0	**1**	0	0	0	0	0	0	0	**1**	0	0	0	0
CNS	0	0	0	0	**1**	0	0	0	**1**	0	0	0	0	0
Rothia dentocariosa	-[c]	0	-	0	-	**1**	-	0	-	**1**	-	0	-	0
Pseudomonas sp[b]	-	0	-	1	-	**1**	-	**1**	-	0	-	0	-	**1**

Table 4.8: Antibiotic Resistance Profiles of Mercury-resistant Bacteria from Children without (-Am) and with (+Am) Amalgam Fillings

a No ampicillin- and high-level gentamicin-resistant bacteria were found

b Penicillin is not used to treat pseudomonal infections, while all Gram-negative bacteria are inherently resistant to erythromycin and vancomycin

c A dash indicates that this organism was not isolated from this patient group

Antimicrobial agent	Viridans streptococci (n=59)			
	Range	MIC_{50} (mg/l)	MIC_{90} (mg/l)	%R
Mercury	32-64	32	64	100
Penicillin	≤0.008-0.5	0.03125	0.125	6
Ampicillin	≤0.008-1	0.0625	0.5	0
Erythromycin	≤0.008-4	0.03125	2	15
Vancomycin	0.125-1	0.5	1	0
Tetracycline	0.0625-32	0.25	16	18
Gentamicin[a]	0.0625-4	2	4	0

Table 4.9: *In vitro* activities of 6 Antimicrobial Agents against Mercury-resistant Oral Streptococci isolated from non-amalgam Patients

Antimicrobial agent	Viridans streptococci (n=66)			
	Range	MIC_{50} (mg/l)	MIC_{90} (mg/l)	%R
Mercury	32-128	32	64	100
Penicillin	≤0.008-0.5	0.03125	0.125	3
Ampicillin	≤0.008-1	0.03125	0.25	0
Erythromycin	≤0.008-8	0.03125	2	21
Vancomycin	0.25-1	0.5	1	0
Tetracycline	0.03125-64	0.5	64	28
Gentamicin[a]	0.0625-8	2	8	0

Table 4.10: *In vitro* activities of 6 Antimicrobial Agents against Mercury-resistant Oral Streptococci isolated from amalgam Patients

[a] Streptococci have inherent resistance to aminoglycosides and low-level resistance to gentamicin is not significant (MIC ≥2–128mg/l). A MIC ≥128mg/l gentamicin is high-level resistance.

Antimicrobial agent	*S. oralis* (n=17)				*S. mitis* (n=9)				Other streptococci (n=33)			
	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R
Mercury	32-64	32	64	100	32-64	32	64	100	32-64	32	32	100
Penicillin	≤0.008-0.5	0.03125	0.125	6	≤0.008-0.125	0.03125	0.125	0.0	≤0.008-0.25	0.0625	0.125	6
Ampicillin	≤0.008-1	0.03125	0.125	0.0	0.016-0.5	0.03125	0.5	0.0	≤0.008-1	0.0625	0.25	0.0
Erythromycin	≤0.008-2	0.03125	1	12	0.016-0.0625	0.03125	0.0625	0.0	≤0.008-4	0.03125	2	21
Vancomycin	0.25-1	0.5	0.5	0.0	0.125-1	0.5	1	0.0	0.125-1	0.5	1	0.0
Tetracycline	0.0625-16	0.25	0.5	6*	0.0625-32	0.5	32	44	0.0625-4	0.25	4	18
Gentamicin[a]	1-4	2	4	0.0	1-2	2	2	0.0	0.0625-4	1	4	0.0

Table 4.11: *In vitro* activities of 6 Antimicrobial agents against Mercury-resistant Oral Streptococci isolated from non-amalgam Patients

Antimicrobial agent	*S. oralis* (n=36)				*S. mitis* (n=7)				Other streptococci (n=23)			
	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R
Mercury	32-64	32	32	100	32-128	64	128	100	32-64	32	32	100
Penicillin	≤0.008-0.125	0.03125	0.125	0.0	0.016-0.5	0.03125	0.5	14	≤0.008-0.25	0.03125	0.0625	4
Ampicillin	≤0.008-0.25	0.03125	0.125	0.0	0.016-1	0.0625	1	0.0	≤0.008-0.25	0.03125	0.25	0.0
Erythromycin	≤0.008-8	0.03125	2	28	≤0.008-1	0.03125	1	14	≤0.008-4	0.03125	1	13
Vancomycin	0.25-1	0.5	1	0.0	0.5-1	1	1	0.0	0.5-1	0.5	1	0.0
Tetracycline	0.125-64	0.5	64	33*	0.0625-64	0.5	64	29	0.03125-64	0.25	32	17
Gentamicin[a]	0.5-8	2	8	0.0	0.5-8	2	8	0.0	0.0625-8	2	8	0.0

Table 4.12: *In vitro* activities of 6 Antimicrobial Agents against Mercury-resistant Oral Streptococci isolated from amalgam Patients

[a] Streptococci have inherent resistance to aminoglycosides and low-level resistance to gentamicin is not significant (MIC ≥2–128). A MIC ≥128μg/ml gentamicin is high-level resistance

* Significant difference using the Chi squared test at the 5% level (P≥0.05)

Chapter Five

A Longitudinal Study –

Resistance of the Commensal Oral Microflora

to Mercury and Antibiotics in Subjects

before and after the placement of Amalgam Fillings

5.0 A Longitudinal Study – Resistance of the Commensal Oral Microflora to Mercury and Antibiotics in Subjects before and after the placement of Amalgam Fillings

5.1 Introduction

As previously discussed, only a few studies have investigated whether the presence of mercury in dental amalgam fillings can act as selective pressure for mercury and antibiotic resistance in commensal bacteria found in the oral and gastrointestinal tract (Summers *et al*, 1993, Österblad *et al*, 1995, Edlund *et al*, 1996). In the few studies that have been undertaken, the majority are cross-sectional studies, where results obtained from subjects with amalgam fillings are compared with results from subjects without amalgam fillings (Österblad *et al*, 1995, Edlund *et al*, 1996). The only reported longitudinal study was carried out by Summers' group in the USA, who found that in cynomolgus monkeys there was a significant increase in mercury resistance in the faecal and oral flora post-amalgam placement (Summers *et al*, 1993).

5.2 Aims

There were three aims to this study. These were:

1. To determine whether placement of mercury amalgam fillings in children's teeth results in an increase in oral bacteria resistant to mercury, penicillin, ampicillin, erythromycin, vancomycin, tetracycline or metronidazole.

2. The second aim was to identify any mercury-resistant bacteria isolated and determine whether there was a difference in the types of organisms isolated from the pre- and post-amalgam saliva and plaque samples.

3. The final aim was to investigate whether the mercury-resistant organisms were also resistant to any of 5 antibiotics and to determine whether there were differences pre- and post-amalgam placement.

5.3 Materials and Methods

5.3.1 Patient Selection and Sampling

Plaque and saliva samples were collected from children (aged 5-18 years) during 3 consecutive visits to the Paediatric department of the Eastman Dental Hospital. These children were healthy and had not taken antibiotics during the preceding

months. They needed conservative dental treatment and had never had amalgam restorations in the past. During the first visit to the clinic the patient was clinically assessed and the child's guardian signed a consent form. One sample of supragingival plaque and one sample of saliva was collected. The sampling technique is described in further detail in Chapter 4.3.1. Further samples were collected one month later, when the patient returned to have the carious lesion restored with amalgam. These samples were taken prior to treatment. The final samples were collected one month after the restoration.

5.3.2 Sample Processing

The fresh saliva and plaque samples were cultured within 30 minutes of arrival at the laboratory, where they were vortexed together and 1 in 10 serially diluted in tryptone soy broth (Chapter 2.3.2). Duplicate 100µl aliquots of dilutions of each sample (10^{-4}, 10^{-5} 10^{-6} and 10^{-7}) were inoculated onto mercury-free Mueller-Hinton agar. Duplicate 100µl aliquots of the undiluted sample and 10^{-1}, 10^{-2} and 10^{-3} dilutions were inoculated onto Mueller-Hinton agar containing 40µM $HgCl_2$. These plates were prepared on the same day that the specimen was collected and kept out of direct light before use. Four plates were used for each dilution, two of which were incubated aerobically and two anaerobically for 48 hours. The samples were also screened for the presence of bacteria resistant to penicillin, ampicillin, erythromycin, tetracycline, vancomycin and metronidazole and each antibiotic was incorporated into Iso-Sensitest agar supplemented with 5% defibrinated horse blood (E&O Laboratories). The concentrations used are shown in the Table 5.1 below:

Antimicrobial	Concentration (mg/l)
Penicillin	4
Ampicillin	8
Erythromycin	1
Vancomycin	8
Tetracycline	8
Metronidazole	32
Kanamycin	50

Table 5.1: Antibiotic Concentration used in the Screening Plates

The dilutions (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were plated in duplicate as 100µl volumes onto the antibiotic plates. Duplicate 100µl aliquots of dilutions of each sample (10^{-4}, 10^{-5} 10^{-6} and 10^{-7}) were inoculated onto antibiotic-free Iso-Sensitest agar. Four plates were used for each dilution – two were incubated at 37°C anaerobically for 48 hours and two aerobically for 48 hours.

Following incubation, colonies growing on the mercury-containing and mercury-free media were enumerated. Colonies growing on the mercury-containing agar showed little morphological differences and therefore at least four colonies from each patient (aerobic and anaerobic) were sub-cultured onto Mueller-Hinton agar containing 40µM $HgCl_2$. After 24-48 hours incubation (aerobic and anaerobic), the organisms were stored at -70°C, for identification and antibiotic-sensitivity testing at a later date. Due to the presence of blood, the organisms growing on the antibiotic-free and antibiotic-containing Iso-Sensitest agar exhibited morphological differences and each colony morphotype was enumerated and a representative colony was sub-cultured to obtain a pure culture.

After 24-48 hours incubation (aerobic and anaerobic), the organisms were stored at -70°C.

5.3.3 Susceptibility Testing

The MIC of mercury and 5 antibiotics were determined for all the mercury-resistant organisms by an agar dilution technique, as described by the British Society for Antimicrobial Chemotherapy (BSAC)(Andrews, 2001b). Both the mercury and antibiotic plates were prepared on the day that they were inoculated. Mueller-Hinton agar was used to determine the mercury MIC, while Iso-Sensitest agar supplemented with 5% defibrinated horse blood was used to determine the MICs of the antibiotics. The stock solutions used are described in Chapter 2.1.4 and the range and breakpoints are shown Table 5.2.

Antimicrobial	Range[a]	Breakpoint[a,b] Gram-positive and *Neisseria* species
Mercury	1-1024	16
Penicillin	0.008-8	0.125
Ampicillin	0.008-8	1
Erythromycin	0.008-8	0.5
Vancomycin	0.0625-16	4
Tetracycline	0.016-128	1

Table 5.2: Range and Breakpoint Concentrations of Antibiotics

[a] The mercuric chloride concentration was measured in μM. The antibiotic concentrations were measured in mg/l

[b] The breakpoint differs according to the organism tested. All *Neisseria* (Gram-negative) are inherently resistant to vancomycin

A Mueller-Hinton plate without mercuric chloride and a 5% defibrinated horse blood Iso-Sensitest plate without antibiotics were prepared to determine whether

these agars could support the growth of the control and test organisms. Before use, the agar plates were dried in a laminar flow cabinet for 20 minutes and kept out of direct light.

The method is described in further detail in Chapter 4.3.4.

5.3.4 Statistical Analysis

The mean, median and interquartile range (IQR) were determined to summarise the distribution pattern of the results obtained. In most cases, the mean and IQR were greater than the median, signifying that the results did not exhibit normal distribution, but were severely positively skewed and therefore could not be subjected to parametric testing (Figures 4.1 & 4.2). The non-parametric Wilcoxon signed-rank test was used to compare the results obtained during visits 1 and 2 (pre-amalgam). This test is based on ranking results in order and allows two groups of the same size (κ) to be compared. The non-parametric Friedman two-way ANOVA (analysis of variance) test was used to analyse the results obtained from the 3 visits. Again, this test is based on ranking results in order, but allows three or more groups of the same size (κ) to be compared. The 5% level of statistical significance was adopted throughout ($P \leq 0.05$).

5.4 Results

The group consisted of 16 patients, mean age 8 years, SD 3.3; range 5-18.4 years. Eleven of the patients were male (69%), while 5 were female (31%).

Prior to looking for potential differences in the proportion of resistant bacteria pre- and post-amalgam, it was important to determine whether there were any differences in the proportion of resistant bacteria during visits 1 and 2 (pre-amalgam). If the proportions of mercury- and antibiotic-resistant bacteria varied considerably between visits 1 and 2 for each patient, it would have been pointless to continue with the longitudinal study and obtain results from post-amalgam patients. Statistically, using the non-parametric Wilcoxon test, the differences between visits 1 and 2 were found not to be significant, with P values ranging from 0.1928-1.

On each visit (visits 1 and 2, pre-amalgam, and visit 3, post-amalgam), 13 of the 16 children (81%) had mercury-resistant bacteria in their oral flora. Ten of the children had mercury-resistant bacteria isolated from their oral flora on all 3 visits. Four children did not have mercury-resistant bacteria during one of the visits, while two children did not possess mercury-resistant bacteria during two visits. All of the children had mercury-resistant bacteria during at least one visit. Table 5.3 shows the proportions of mercury-resistant bacteria, expressed as percentages of the total viable aerobic and anaerobic counts, present in the samples. Under aerobic conditions of cultivation, the median proportions of mercury-resistant bacteria were greater amongst subjects post-amalgam (visit 3) than pre-amalgam (visits 1 and 2). However, using anaerobic cultivation, the median proportion of mercury-resistant bacteria was far greater with visits 2 (pre-amalgam) and 3 (post-amalgam) than visit 1 (pre-amalgam). The mean and IQR for both incubation conditions and for all 3 visits were greater than the

correlating median, suggesting that the results were not normally distributed and positively skewed (skewed to the right). However, unlike the cross-sectional study results (Chapter 4.4), which were transformed logarithmically to allow parametric testing, these results could not be transformed, since transformation is not suitable for small data sets (only 16 patients were sampled in this study). Therefore, a non-parametric test was conducted in which the results from the 3 visits were all compared. The Friedman two-way ANOVA test fits the criteria. Only the anaerobic results were analysed because most of the bacteria of interest were either obligate anaerobes or facultative anaerobes. These differences between the 3 groups were not significant at the 5% level ($P \leq 0.05$) as $P=0.802$.

Bacteria resistant to penicillin were isolated from 94%, 100% and 100% of the 16 children on visits 1, 2 and 3 respectively. When expressed as a proportion of the total aerobic count, the median proportions of mercury-resistant bacteria were greater amongst subjects during visit 1 (pre-amalgam) than visits 2 (pre-amalgam) and 3 (post-amalgam)(Table 5.4). However, when expressed as a proportion of the total anaerobic count, the median proportions of mercury-resistant bacteria were very similar during the 3 visits. As with the mercury-resistant proportions, the mean and IQR for both incubation conditions and for all 3 visits were greater than the correlating median, suggesting that the results were not normally distributed and positively skewed. Again, the results based on the total anaerobic count were analysed using the two-way Friedman test and the results were found to be not significant ($P=0.549$).

Ampicillin-resistant bacteria were isolated from 94%, 88% and 100% of the 16 children on visits 1, 2 and 3 respectively. Table 5.5 shows that the mean and IQR were greater than the correlating median, signifying non-normal distribution. Therefore, the results based on the total anaerobic count were analysed using the Friedman test and found to be not significant (P=0.936).

Erythromycin-resistant bacteria were isolated from all of the children on each of the 3 visits. The mean, median and IQR were calculated on the basis of the total aerobic and anaerobic counts (Table 5.6). For some visits the mean, median and IQR were found to be very similar, indicating that these results were normally distributed and should be analysed using a parametric test. However, for some visits, the mean and IQR were greater than the median, indicating that these results were positively skewed and could be analysed using a non-parametric test. As a result, the non-parametric Friedman test was used as previous results in this study had been analysed using this test. There was no significant difference in the proportions of erythromycin-resistant bacteria isolated on the 3 visits (P=0.819).

Bacteria resistant to tetracycline were isolated from all 16 of the children on each of the 3 visits. The mean and IQR for both incubation conditions and for all 3 visits were greater than the correlating median, suggesting that the results were not normally distributed and positively skewed (Table 5.7). Using the Friedman test, the results obtained on each visit were found to be not significantly different (P=0.766).

Vancomycin-resistant bacteria were isolated from all of the children on each of the 3 visits (Table 5.8). However, all Gram-negative bacteria are inherently resistant to vancomycin and it is probable that these vancomycin-resistant organisms were Gram-negative organisms. It would be necessary to Gram stain these organisms to determine whether resistance is inherent or acquired. However, due to time constraints, this was not possible. Using the Friedman test, there was no significant difference in the proportions of vancomycin-resistant bacteria isolated on the 3 visits (P=0.344).

No metronidazole-resistant organisms were isolated from any of the 16 children. Although initially organisms were isolated on the metronidazole-containing agar, after retesting by growing the bacteria under aerobic and anaerobic conditions, none of the isolates were found to be obligate anaerobes.

All the mercury-resistant organisms were identified to the genus level, and, if possible, to the species level, and the MIC of $HgCl_2$ was determined for each. Table 5.9 shows the number of mercury-resistant species isolated during each visit. There were no obvious differences between the three visits in terms of the number of mercury-resistant species isolated. Four patients (25%) from visit 1 and three patients (20%) from visits 2 and 3 did not have any mercury-resistant bacteria. Fewer mercury-resistant species were isolated from patients during visit 1 (pre-amalgam). During visit 1, of the patients that harboured mercury-resistant bacteria, only 1 to 3 different species were isolated from each patient. During visit 3 (post-amalgam), of the patients that harboured mercury-resistant bacteria, between 1 and 4 different mercury-resistant species

were isolated. However, during visit 2 (pre-amalgam) between 1 and 5 different mercury-resistant organisms were isolated from patients that harboured mercury-resistant organisms.

A total of 88 different mercury-resistant organisms were isolated from the study. Twenty one, 34 and 33 mercury-resistant organisms were isolated on visits 1, 2 and 3 respectively (Table 5.10). Eighty of the 88 (91%) mercury-resistant bacteria were streptococci. Of the mercury-resistant bacteria isolated from the children pre-amalgam, 91% (50 out of 55) were streptococci. Indeed, all of the mercury-resistant organisms isolated during the first visit were identified as streptococci. Similarly, 91% (30 out of 33) of the mercury-resistant organisms isolated from the children post-amalgam were identified as streptococci. Of those streptococci that could be easily identified biochemically and by 16S rRNA sequencing, the majority were *S. oralis* (32%). In the pre-amalgam group, 24% (13 out of 55) of the organisms were *S. oralis*, while in the post-amalgam group, 45% (15 out of 33) of the organisms were *S. oralis*. However, using the Chi square test, this difference was narrowly not significant ($\chi^2_{(1)}$=3.576, P=0.0586). *Streptococcus mitis* was the second most commonly isolated mercury-resistant streptococcal species (25%). Twenty nine percent (16 out of 55) of the mercury-resistant organisms isolated from visits 1 and 2 were identified as *S. mitis*, while during visit 3, 18% (6 out of 33) of the bacteria were *S. mitis*. However, using the Chi square test, these differences were not significant ($\chi^2_{(1)}$=0.792, P=0.373).

As discussed, all of the mercury-resistant organisms isolated during visit 1 were identified as streptococci. However, the mercury-resistant bacteria isolated from visits 2 and 3 showed a greater diversity with a variety of genera exhibiting mercury resistance. The other mercury-resistant organisms isolated included the Gram-positive bacteria coagulase-negative staphylococci (*Staph. hominis, Staph. epidermidis* and unidentified CNS) and *R. dentocariosa* and a *Neisseria* species. These organisms will be discussed later in this chapter. No mercury-resistant obligate anaerobes were isolated from either pre-amalgam or post-amalgam patients.

Once identified, the mercury-resistant bacteria were tested for their level of resistance to mercury, by determining the MIC of $HgCl_2$. The majority of organisms had an MIC of 32μM, although some had an MIC of 64μM (Table 5.11). Only one organism, a *Staph. hominis* from a patient post-amalgam, displayed an MIC of 128μM.

All of the mercury-resistant bacteria isolated were tested for their susceptibility to 5 commonly used antibiotics by determining the MIC of the antibiotic. Fifteen (27%) and 13 (40%) of the mercury-resistant bacteria isolated from the pre- and post-amalgam patients were also resistant to at least one of the antibiotics tested (Table 5.12). Resistance to metronidazole was not tested as all of the mercury-resistant organisms were identified as facultative anaerobes. None of the mercury-resistant bacteria exhibited resistance to ampicillin. The mercury-resistant organisms were most often resistant to erythromycin (25%) and to a lesser extent, tetracycline (10%). Penicillin resistance was occasionally

encountered in these organisms (7%). Four isolates, three from patients pre-amalgam and one from a post-amalgam patient, were resistant to two antibiotics. These isolates were either resistant to both penicillin and erythromycin, penicillin and tetracycline or tetracycline and erythromycin (Table 5.13). A *Neisseria* species isolated from a patient pre- and post-amalgam was resistant to four antibiotics – penicillin, vancomycin and erythromycin and tetracycline (Table 5.14). However, *Neisseria* are Gram-negative and therefore inherently resistant to vancomycin, so it is only the penicillin, erythromycin and tetracycline resistances that are of significance.

Tables 5.15, 5.16 and 5.17 show the MIC range, MIC_{50} and MIC_{90} of the 5 antibiotics tested and the percentage of resistance for all the streptococci isolated. All isolates from the pre- and post-amalgam patients were sensitive to ampicillin (MIC_{90} = 0.125mg/l). All isolates displayed vancomycin sensitivity with MIC_{50} and MIC_{90} values of 0.5 and 1mg/l for both pre- and post-amalgam. Pre-amalgam, the rank order of susceptibility for the viridans streptococci was: vancomycin = ampicillin (100%) > penicillin (97%) > tetracycline (93%) > erythromycin (81%). The same pattern was also observed post-amalgam: vancomycin = ampicillin = penicillin (100%) > tetracycline (93%) > erythromycin (73%). For both the pre- and post-amalgam patients, erythromycin seemed to be the least active, with 21 and 27% resistance rates.

Tables 5.18, 5.19 and 5.20 show the MIC range, MIC_{50} and MIC_{90} of the 5 antibiotics tested and the percentage of resistant strains for *S. oralis*, *S. mitis* and *Streptococcus* species (including the unspeciated streptococci, *S. salivarius*,

S. parasanguinis, *S. sanguinis*, *S. dysgalactiae*, *S. infantis*, *S. peroris* and *S. vestibularis*). The MIC range of vancomycin for the streptococci was narrow, with MIC_{90}s of 0.5 and 1mg/l and all of the organisms tested were sensitive to vancomycin. In contrast, the MIC ranges of the other antibiotics, especially for erythromycin and tetracycline were much larger. Due to the large MIC range for these antibiotics, the MIC_{50} and MIC_{90} values also varied quite considerably. The results for the coagulase-negative staphylococci, *Staph. hominis*, *Staph. epidermidis*, *R. dentocariosa* and the *Neisseria* species are not listed in the table because of the limited number of these organisms isolated.

Four mercury-resistant staphylococci were isolated in this study and identified to species level using 16S rRNA sequencing. Of the organisms isolated, one was a *Staph. hominis*, one was a *Staph. epidermidis* and 2 were unidentifiable using 16S rRNA and therefore referred to as coagulase-negative staphylococci. The MIC of $HgCl_2$ for the coagulase-negative staphylococci and the *Staph. epidermidis* was 32μM to mercury, while that for the *Staph. hominis* was 128μM, indicating a high resistance to $HgCl_2$ (Table 5.11). This organism was isolated from a patient post-amalgam. All the staphylococci isolated were moderately resistant to the antibiotics tested. The *Staph. hominis* showed resistance to penicillin (MIC=0.5mg/l) and tetracycline (MIC=64mg/l), while one of the coagulase-negative staphylococci (post-amalgam) was resistant to erythromycin (MIC=1mg/l)(Table 5.14). Ampicillin and vancomycin resistance was not observed in any of the staphylococci.

In this study, two mercury-resistant *R. dentocariosa* strains were isolated from patients without amalgam fillings. These organisms were identified using 16S rRNA sequencing. The MIC of mercury for both organisms was 32μM (Table 5.11). Both isolates were fully susceptible to the 5 antibiotics tested (Tables 5.13 and 5.14).

The only mercury-resistant Gram-negative organism isolated in this study was a *Neisseria* species which was isolated from a patient during visits 2 and 3 (pre- and post-amalgam). This organism was tentatively identified as *Neisseria sicca* by 16S rRNA gene sequencing. The MIC of $HgCl_2$ was 32μM for this organism (Table 5.11). It was also resistant to penicillin (MIC=2mg/l), erythromycin (MIC=4mg/l), tetracycline (MIC=2mg/l) and vancomycin (MIC>16mg/l).

5.5 Discussion

Since the normal flora of individuals can vary considerably from individual to individual, this study, like all longitudinal studies, allows each subject to act as their own longitudinal control. Few studies have looked at the prevalence or persistence of mercury- and antibiotic-resistant organisms in the oral cavity, so before investigating whether the presence of amalgam results in an increase in the proportion of mercury- or antibiotic-resistant bacteria, it was important to determine whether the number of mercury- and antibiotic-resistant bacteria remained constant during the pre-amalgam two month period. If the proportion of mercury- and antibiotic-resistant organisms varied considerably for each patient

between visits 1 and 2, it would have been pointless to have continued the study and collect post-amalgam samples. Fortunately, using the Wilcoxon Signed rank test, any potential differences observed during visits 1 and 2 were found not to be significant, with all the P values greater than 0.05.

The first aim of this study was to explore whether the placement of amalgam fillings resulted in an increase in mercury-resistant bacteria in the oral flora of children. Similar numbers of children, pre- and post-amalgam, harboured mercury-resistant bacteria in their oral flora (81%). These results were similar to those found in the cross-sectional study (Chapter 4) in which 71% of children without amalgam fillings and 78% of children with amalgam fillings were found to harbour mercury-resistant bacteria in their oral flora.

Previous studies have shown that newly-placed amalgam releases greater levels of mercury vapour than older amalgam that has tarnished (Brune, 1981). Therefore, if the theory that amalgam results in an increase in mercury and antibiotic resistance is valid, one would expect to see a change in the proportion of resistant bacteria shortly after the amalgam restoration. However, in this study, the proportion of mercury-resistant bacteria had not significantly changed one month after placement of the mercury amalgam fillings.

There was also a failure to detect any change in the proportion of oral bacteria resistant to penicillin, ampicillin, erythromycin, vancomycin or tetracycline. All the children in the study harboured erythromycin-, vancomycin- and tetracycline-resistant bacteria in their oral flora and a large proportion of the

children also harboured penicillin- and ampicillin-resistant bacteria. Statistically, the prevalence of antibiotic-resistant bacteria pre- and post-amalgam was not a significant difference.

Various studies have incorporated antibiotics into agar to select for, and to determine the proportion of antibiotic-resistant commensal bacteria in the oral and faecal flora. It is particularly common in longitudinal studies that look at the effect on resistance of taking a course of antibiotics, where the proportion of resistant bacteria is determined prior to and after the administration of the antibiotic. Most of these studies have found that most individuals carry antibiotic-resistant oral streptococci in their mouths prior to taking antibiotics (Maskell *et al*, 1990, Sefton *et al*, 1999). An investigation by Maskell found that, in a preliminary study of 45 subjects, 44 harboured oral streptococci resistant to 1mg/l erythromycin and 30 yielded organisms resistant to 64mg/l of the antibiotic (Maskell *et al*, 1990). However, in general, this constituted only 0.01% (0.00001-0.18%) of the total streptococci isolated. In the second part of the study, prior to antibiotic administration, it was found that of the 16 subjects tested, all harboured bacteria resistant to 1mg/l erythromycin. However, the proportion of resistant oral streptococci constituted less than 1.5% of the total streptococci. Finally, it was found that 87% of the subjects harboured bacteria resistant to 4mg/l erythromycin and 50% yielded bacteria resistant to 64mg/l of the antibiotic. In addition 81% of the subjects harboured bacteria resistant to 1mg/l amoxycillin. Twenty five percent of subjects had streptococci resistant to 4mg/l amoxycillin. Finally, after the administration of erythromycin, the proportion of erythromycin-resistant organisms increased substantially.

An investigation by Sefton also showed that of 16 subjects investigated, all harboured streptococci resistant to 1mg/l erythromycin (Sefton, 1999). Similarly to Maskell's results, the proportion of streptococci resistant to 1mg/l was less than 1.5% of the total cultivable streptococcal population (Maskell *et al*, 1990). Eighty eight percent and 38% of individuals yielded streptococci resistant to 4mg/l and 64mg/l erythromycin respectively. As with Maskells' study, two days after the administration of a macrolide antibiotic the proportion of erythromycin-resistant streptococci increased.

In the present study, prior to amalgam placement, 94% of patients during visit 1 and 88% of patients during visit 2 harboured bacteria resistant to 8mg/l ampicillin. The proportion of bacteria resistant to ampicillin ranged from 0-100% of the total cultivable flora. The sample that contained 100% ampicillin-resistant organisms was taken pre-amalgam and seems to be erroneous, as the median from the sampling group was 6.6. However, such high levels of resistance have also been observed in a study by Koh where a patient with a history of rheumatic fever had 95% of oral streptococci resistant to 1mg/l amoxycillin (Koh *et al*, 1986). In the present study, one month after the placement of amalgam, 100% of patients yielded bacteria resistant to 8mg/l ampicillin. Similar to Maskell's and Sefton's work, all 16 patients in the present study harboured bacteria resistant to 1mg/l erythromycin. However, the present study found that the proportion of erythromycin-resistant bacteria ranged from 0.42-100% (median 19.6%) of the total bacterial count, while the earlier studies found that less than 1.5% of the total streptococci were resistant to the

same concentration of erythromycin (1mg/l). These differences may be accounted for by the fact that in the present study the erythromycin-resistant bacteria were isolated on non-selective media while Maskell and Sefton isolated their resistant bacteria on erythromycin-containing Mitis Salivarius Agar, which is selective for viridans streptococci. The majority of erythromycin-resistant bacteria isolated in the present study may be species other than streptococci. These erythromycin-resistant bacteria were not identified due to time limitations.

Two other mercury resistance studies have looked at the incidence of antibiotic resistance directly by incorporating antibiotics into the agar. Edlund carried out a cross-sectional study, while Summers' study was longitudinal (Summers *et al*, 1993, Edlund *et al*, 1996). Edlund incorporated 16mg/l ampicillin and 4mg/l erythromycin into various types of agar. In both the non-amalgam and amalgam groups, the proportion of organisms resistant to ampicillin was very low and the median values for both groups were zero. The erythromycin results were not well reported, but it can be concluded that the group found that erythromycin resistance did not vary significantly between the 2 groups. When analysing the groups together, the median percentage (range) of the oral microflora resistant to erythromycin was 1.4% (0-52%). However, the paper does not report on the number or percentage of individuals harbouring erythromycin-resistant bacteria.

The longitudinal study carried out by Summers and co-workers found that even prior to amalgam installation, oral streptococci resistant to 15mg/l tetracycline were present. While tetracycline resistance persisted, it did not fluctuate with the

placement or removal of the amalgam fillings. The percentage of streptococci resistant to 15mg/l tetracycline ranged from 0-20%. In the present study all 16 patients harboured bacteria resistant to 8mg/l tetracycline, with a median value of 29.9% of the total cultivable microflora. However, unlike in the present study where the majority of pre-amalgam patients harboured ampicillin-resistant organisms, Summers' group did not isolate any ampicillin-resistant streptococci on initial screening. Summers' reported that ampicillin-resistant streptococci were 'later' isolated at a low incidence in a few gingival scrapings, but did not state whether this was pre-amalgam, post-amalgam or post amalgam replacement. The differences between the two groups may be explained in that Summers' group incorporated 25mg/l ampicillin into Mitis Salivarius agar, while the present study incorporated only 8mg/l into Iso-Sensitest agar.

The second aim of the study was to identify the mercury-resistant bacteria and to determine whether there were differences in the genera and species isolated from the pre- and post-amalgam samples. Table 5.9 shows the number of different species isolated at each visit. In general, the number of different species isolated did not differ greatly pre- and post-amalgam. Table 5.10 shows that the majority of mercury-resistant organisms were streptococci. Both pre- and post-amalgam, 91% of the mercury-resistant organisms were streptococci. *Streptococcus mitis* was more commonly isolated from patients pre-amalgam, while post-amalgam, *S. oralis* was the most common *Streptococcus* species. This pattern was also observed in the cross-sectional study (Chapter 4). In children, these species of streptococci, plus *S. salivarius*, are the most predominant streptococci found in the oral flora (Lucas *et al*, 2000). Lyttle and Bowden have shown that strains of

S. oralis and *S. mitis* can adapt to a high concentration of mercury by enrichment culture (Lyttle & Bowden, 1993b).

The other species of mercury-resistant organisms (*Staphylococcus*, *Neisseria* and *Rothia*) isolated in this study will be discussed later in this chapter.

The final aim of this study was to investigate whether the mercury-resistant organisms were also resistant to any of 5 antibiotics and to determine whether there were differences pre- and post-amalgam. These antibiotics were penicillin, ampicillin, erythromycin, tetracycline and vancomycin. Metronidazole was not tested as all of the mercury-resistant organisms were facultative anaerobes. None of the streptococci were resistant to vancomycin or ampicillin. Three percent of the pre-amalgam isolates were resistant to penicillin, while none of the post-amalgam streptococci were resistant to penicillin. The rank order of susceptibility for the mercury-resistant viridans streptococci isolated both from pre- and post-amalgam samples was: vancomycin = ampicillin > penicillin > tetracycline > erythromycin. However, in the cross-sectional study, the rank order of susceptibility for the mercury-resistant viridans streptococci isolated samples taken from patients' with and without amalgam was: vancomycin = ampicillin > penicillin > erythromycin > tetracycline. In the longitudinal study, more isolates were resistant to erythromycin than to tetracycline, while in the cross-sectional study, more isolates were resistant to tetracycline than to erythromycin. In the cross-sectional study, eighteen percent of the isolates from the non-amalgam group and 28% of the isolates from the amalgam group were resistant to tetracycline. However, in the longitudinal only 7% of the isolates

from both the pre- and post-amalgam samples were resistant to tetracycline. In the cross-sectional study, fifteen percent of the isolates from the non-amalgam group and 21% of the isolates from the amalgam group were resistant to erythromycin. Nineteen percent of pre-amalgam and 27% of post-amalgam streptococci were resistant to erythromycin. Similar to the longitudinal study, all the mercury-resistant viridans streptococci isolated in the cross-sectional study (Chapter 4) were found to be sensitive to vancomycin and ampicillin. In addition, a similar proportion of organisms were resistant to penicillin (6% non-amalgam and 3% amalgam).

As discussed, this study and many others have shown that antibiotic and mercury resistance is commonly encountered in viridans streptococci (Summers *et al*, 1993, Jones and Pfaller, 2000, Johnson *et al*, 2001). There is substantial evidence to suggest that viridans streptococci can act as genetic reservoirs and transfer genetic information to transient bacteria as they make their way through the mouth, a principal entry point for a wide variety of bacteria (Cvitkovitch, 2001, Bryskier, 2002,). Of particular concern is transfer of antibiotic resistance from viridans streptococci to *S. pneumoniae*, which are naturally transformable (Dowson *et al*, 1993). Many viridans streptococci such as *S. mutans*, *S. mitis*, *S. oralis*, *S. sanguinis* and *S. infantis* are also naturally competent (Gaustad, 1985, Ronda *et al*, 1988, Whatmore *et al*, 2000, Cvitkovitch, 2001, Ween *et al*, 2002). These organisms possess the *com* operon which contains the three genes, *comC*, *comD* and *comE*, which encode proteins involved in regulating competence (Whatmore *et al*, 2000).

Only one other longitudinal study has investigated whether the placement of amalgam fillings results in an increase in mercury and antibiotic resistance in the commensal flora. This study, by Summers, found an increase in the numbers of mercury-resistant oral and faecal bacteria following amalgam placement in Cynomolgus monkeys (Summers *et al*, 1993). After 8 weeks, the amalgam fillings were replaced with glass ionomer fillings, resulting in a sudden increase in the number of mercury-resistant Enterobacteriaceae and oral streptococci. However, a peak in mercury resistance in enterococci was not seen until 5 weeks after the amalgam was removed. The study found that removal of amalgam fillings resulted in an increase of Hg^{2+} in the faeces of the monkeys. In one monkey, the level of Hg^{2+} rose to 288μg/g. In contrast, the average Hg^{2+} in faeces while the amalgam fillings were in place was 2-5μg/g. In this study, the monkeys received a massive number of amalgam fillings. Each monkey was given 16 small, occlusal (biting) surface amalgam fillings containing between 93 and 100mg of mercury. In the present study, the mean number of fillings per child post-amalgam was only six. In Summers' study, the fillings were placed on the biting surface of the tooth, an area where abrasion, common through eating and bruxism (grinding), occurs. This is likely to have resulted in a greater release of mercury than would be observed with "randomly" placed fillings. The group found that 2-5μg/g mercury was excreted (in faeces) daily. In contrast, the study by Österblad found that only 1μg/g mercury was excreted daily from adults with fillings and 0.1μg/g mercury was found in the faeces of adults that had their amalgam removed (Österblad *et al*, 1995). However, there is no reference to the number of fillings present or removed during the study. Edlund found that subjects with amalgam fillings (mean 19 surfaces) excreted only 0.7μg/g mercury

in their faeces per day (Edlund *et al*, 1996). Another shortcoming with Summers' study is that the group worked with only 6 monkeys, which is a very small sample number. Although longitudinal studies require fewer subjects than cross-sectional studies, small sample groups can result in sampling errors and bias and the study findings can be assumed to be less reliable.

Four mercury-resistant staphylococci were isolated in this study and identified as *Staph. hominis*, *Staph. epidermidis* and 2 unidentifiable coagulase-negative staphylococci. The *Staph. hominis* was very resistant to mercury with an MIC of 128μM and was isolated from a patient post-amalgam. This organism also showed high-level resistance to tetracycline (MIC=64mg/l). The breakpoint for tetracycline is 1mg/l. Many studies have looked at mercury resistance in *Staph. aureus*, although few have considered other species of staphylococci (Dyke *et al*, 1970, Hall, 1970a, 1970b, Groves *et al*, 1975, Porter *et al*, 1982, Witte *et al*, 1986). Groves compared antibiotic- and heavy metal resistance in *Staph. aureus* and coagulase-negative staphylococci isolated from the nasopharynx and skin of rural and urban populations exposed to either antibiotics or heavy metals (Groves *et al*, 1975). In a rural population that had been exposed to a methylmercury fungicide, a significant increase in mercury resistance in staphylococci was not detected. However, the study found that *Staph. aureus* was less susceptible to mercury than coagulase-negative organisms. Of the mercury-resistant coagulase-negative staphylococci isolated, very few were also resistant to the two antibiotics tested - penicillin and tetracycline. Interestingly, Groves' study also found that, in *Staph. aureus*, exposure to antibiotics led to an

increase in mercury-, copper- and cadmium resistance. This was not seen in the coagulase-negative strains.

In this study, two mercury-resistant *Rothia dentocariosa* strains were isolated from patients without amalgam fillings. This organism is a normal inhabitant of the oral cavity and is present in human saliva and supragingival plaque (Salamon and Prag, 2001). Up to now, few authors have looked at mercury resistance in this organism although studies have found the *merA* gene in micrococci, also a member of the *Micrococcaceae* (Silver and Misra, 1988, Bogdanova and Mindlin, 1991). The only reports of antimicrobial susceptibility patterns of *Rothia dentocariosa* are cases in which the organism has caused an infection in predisposed individuals (Kong *et al*, 1998, Salamon and Prag, 2001). Authors have reported susceptibility to penicillin, ampicillin, erythromycin and vancomycin (Kong *et al*, 1998, Salamon and Prag, 2001). In the present study, all isolates were fully sensitive to these antibiotics.

Only one Gram-negative Hg-resistant organism was isolated in this study and was tentatively identified as *Neisseria sicca*. *Neisseria sicca* is part of the commensal bacterial flora of the human upper respiratory tract and shares this ecological niche with *Neisseria meningitidis*. Like other *Neisseria* species, *N. sicca* is naturally transformable and can acquire resistance genes from other organisms in the environment. It is possible that antibiotic resistance genes can transfer from *N. sicca* to *N. meningitidis*. The transfer of penicillin resistance genes, from *N. sicca* to *N. meningitidis* is of concern as penicillin is the drug of choice for the treatment and prophylaxis of meningococcal mengitidis. However, there are few

studies on the susceptibility patterns of *N. sicca* to the antimicrobial drugs frequently used in the treatment and prophylaxis of meningococcal disease. This is because, to date, commensal *Neisseria* species are regarded as non-pathogenic saprophytes. However, cases have been reported in which these commensals have been isolated from blood cultures of patients with septicaemia and endocarditis (Feder & Garibaldi, 1984, Szabo *et al*, 1990, Heiddel *et al*, 1993). The majority of these patients had a predisposing factor such as rheumatic heart disease or a history of intravenous drug abuse. *Neisseria sicca* has also been reported as a cause of mengitidis, pneumonia, inflammatory spondylitis, osteomyelitis and urethritis (Heiddel *et al*, 1993). To date, few authors have reported on mercury resistance in *Neisseriaceae*. One study by Riley and Taylor looked at the susceptibility of 56 *Moraxella* (*Branhamella*) *catarrhalis* strains and 10 *Neisseria* species to11 heavy metals, including mercury (Riley & Taylor, 1989). None of the *Neisseria* species were identified as *N. sicca.* The group determined susceptibility by agar dilution, using GC agar supplemented with 0.5% yeast extract and found that, with all the metals, the *M. catarrhalis* strains were less susceptible than the *Neisseria* species. Organisms able to grow on agar containing 100μM (370mg/l) $HgCl_2$ were regarded as mercury-resistant and the group found that 94.6% of the *M. catarrhalis* tested were resistant. The authors concluded that these organisms were probably intrinsically resistant to the metals or contained plasmids that harboured heavy metal resistance genes. In the present study, the mercury-resistant *N. sicca* was also resistant to penicillin, erythromycin, tetracycline and vancomycin. However, all members of the *Neisseriaceae* are intrinsically resistant to vancomycin. Therefore this organism was sensitive to

ampicillin and resistant to penicillin, erythromycin and tetracycline. As discussed, there are very few reports on antimicrobial susceptibility patterns of infections caused by *N. sicca*, however, in one case of *N. sicca* endocarditis, the organism was found to be sensitive to penicillin, plus ampicillin, erythromycin and tetracycline (Heiddel *et al*, 1993).

In this study and the cross-sectional study (Chapter 4), mercury-resistant obligate anaerobes were not isolated from any of the patients. However, this does not mean that mercury-resistant anaerobes do not exist. Several groups have reported mercury-resistant *Bacteroides* species isolated from human and primate faecal samples (Avila-Campos *et al*, 1991a, Edlund *et al*, 1996). Avila-Campos showed that mercury resistance was stable after 10 successive subcultures. However, the study was unable to show whether resistance was plasmid-mediated. Mercury-resistant *Bacteroides ruminicola* and *Clostridium perfringens* have also been isolated from clinical and sewage samples (Rudrik *et al*, 1985). Rudrik isolated the organisms on cysteine-free agar, since it is known that mercury chelates to the sulphur found in this amino acid, which could result in the isolation of false-positives. However, another study led by Summers and Sugarman, found that sulfhydryl groups in this amino acid are necessary for mediating mercury resistance by maximising the activity of mercury reductase, the enzyme that reduces Hg^{2+} to Hg^{0} (Summers and Sugarman 1974). It is possible that the agar used in the present study contained insufficient sulfhydryl groups for the mercury reductase in anaerobic organisms to work and therefore the organisms were unable to grow on the mercury-containing agar.

In conclusion, this study shows that mercury and antibiotic resistance in the oral flora is widely distributed among healthy children and that dental amalgam alone does not appear to be a key factor in promoting spread. This was also observed in the cross-sectional study (Chapter 4). However, in the cross-sectional study, it was difficult to control other factors and variables that may select for resistance. These include traces of antibiotics and mercury in food, such as meat, fish, milk, vegetables and fruit (Corpet, 1993). Although these variables were also present in longitudinal studies, they did not pose such a problem as each subject acts as their own longitudinal control.

The longitudinal study showed that mercury resistance was present in a variety of different organisms found in the oral flora such as *Streptococcus*, *Staphylococcus*, *Rothia* and *Neisseria*. Akin to the cross-sectional study, *S. oralis* was the most commonly isolated mercury-resistant *Streptococcus* species. Twenty four percent of the mercury-resistant organisms isolated during the pre-amalgam visits were *S. oralis*, while in the post-amalgam group 45% of the organisms were *S. oralis*. However, unlike the cross-sectional study, these results were narrowly not significant.

Other differences between the cross-sectional and longitudinal study were also observed. An important difference between the longitudinal and cross-sectional studies was that in the longitudinal study more mercury-resistant organisms were resistant to erythromycin (25.0%) than tetracycline (10.2%). In the cross-sectional study, more of the mercury-resistant organisms were resistant to tetracycline (21.6%) than erythromycin (17.3%).

This study highlights that, like the cross-sectional study, viridans streptococci are the group that exhibits most resistant types to commonly used antimicrobial agents, suggesting the need for comprehensive surveillance programmes to investigate and monitor resistance in commensal organisms (Bax *et al*, 2000).

	Proportion of aerobic count (%)			Proportion of anaerobic count (%)		
	without amalgam		with amalgam	without amalgam		with amalgam
	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
Range	0-9.57	0-2.66	0-1.585	0-4.44	0-2.33	0-4.56
Mean	0.8569	0.2299	0.14448	0.8192	0.2999	0.4111
Median	0.0038	0.004	0.0095	0.0006	0.025	0.029
Inter-quartile range	0.2725	0.125	0.0258	1.1638	0.185	0.1586
Friedman test P value					P=0.802	

Table 5.3: Proportions of Mercury-resistant Microbes in the oral Microflora of Children with and without Amalgam Fillings

	Proportion of aerobic count (%)			Proportion of anaerobic count (%)		
	without amalgam		with amalgam	without amalgam		with amalgam
	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
Range	0-2.58	0-4.76	0-1.82	0-18.15	0.054-17.65	0.003-27.02
Mean	0.6465	0.6245	0.3487	3.1491	4.3883	4.2446
Median	0.2	0.04	0.0575	1.775	1.48	1.415
Inter-quartile range	0.5465	0.719	0.2314	3.125	7.07	5.34
Friedman test P value					P=0.549	

Table 5.4: Proportions of Penicillin-resistant Microbes in the Oral Microflora of Children with and without Amalgam Fillings

	Proportion of aerobic count (%)			Proportion of anaerobic count (%)		
	without amalgam		with amalgam	without amalgam		with amalgam
	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
Range	0-100	0-21.24	0-5.17	0-6.86	0-4.43	0-2.19
Mean	6.6577	1.7654	0.434	1.7011	0.905	5.813
Median	0.03	0.01	0.0199	0.15	0.21	0.455
Inter-quartile range	0.6825	0.42	0.225	2.4125	0.882	0.6125
Friedman test P value					P=0.936	

Table 5.5: Proportions of Ampicillin-resistant Microbes in the Oral Microflora of Children with and without Amalgam Fillings

	Proportion of aerobic count (%)			Proportion of anaerobic count (%)		
	without amalgam		with amalgam	without amalgam		with amalgam
	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
Range	0.42-56.38	4.47-100	5.61-38.84	5-64	2.79-100	6.95-46.83
Mean	22.3956	22.58	16.4169	28.1944	28.922	25.1138
Median	22.705	14.42	14.265	20.76	22.54	24.435
Inter-quartile range	21.0825	15.355	10.6125	28.225	27.935	14.6275
Friedman test P value					P=0.819	

Table 5.6: Proportions of Erythromycin-resistant Microbes in the Oral Microflora of Children with and without Amalgam Fillings

	Proportion of aerobic count (%)			Proportion of anaerobic count (%)		
	without amalgam		with amalgam	without amalgam		with amalgam
	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
Range	0.015-18.51	0.011-17.34	0.2-14.10	0.02-17.56	0.13-23.94	0.27-14.54
Mean	4.2066	4.2167	4.8963	4.1938	7.218	4.43
Median	2.05	2.39	2.345	3.36	3.81	2.235
Inter-quartile range	3.27	4.77	6.7425	6.2975	10.72	3.045
Friedman test P value					P=0.766	

Table 5.7: Proportions of Tetracycline-resistant Microbes in the Oral Microflora of Children with and without Amalgam Fillings

	Proportion of aerobic count (%)			Proportion of anaerobic count (%)		
	without amalgam		with amalgam	without amalgam		with amalgam
	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
Range	4.6-34.27	2.04-26.61	4.12-31.83	2.66-84.44	2.8-58.89	4.97-100
Mean	18.0163	12.1187	11.3056	17.7066	16.1547	26.4856
Median	16.38	9.78	10.195	9.64	11.11	18.035
Inter-quartile range	11.1275	9.08	4.79	13.895	17.34	14.895
Friedman test P value					P=0.344	

Table 5.8: Proportions of Vancomycin-resistant Microbes in the Oral Microflora of Children with and without Amalgam Fillings

Number of mercury-resistant organisms	Number of patients		
	Pre-amalgam		Post-Amalgam
	Visit 1	Visit 2	Visit 3
0	4 **(25.0)**	3 **(33.0)**	3 **(18.8)**
1	5 **(31.3)**	1 **(6.3)**	2 **(12.5)**
2	5 **(31.3)**	4 **(25.0)**	5 **(31.3)**
3	2 **(12.5)**	4 **(25.0)**	3 **(18.8)**
4	0 **(0.0)**	2 **(12.5)**	3 **(18.8)**
5	0 **(0.0)**	1 **(6.3)**	0 **(0.0)**

Table 5.9: Number **(%)** of Patients with a given number of Mercury-resistant Species

	No. (%) isolates from children		
	Pre Amalgam		Post Amalgam
Organism	Visit 1	Visit 2	Visit 3
Streptococcus oralis	6 **(29)**	7 **(21)**	15 **(45)**
Streptococcus mitis	7 **(33)**	9 **(27)**	6 **(18)**
Streptococcus salivarius	0 **(0)**	3 **(9)**	1 **(3)**
Streptococcus sanguis	2 **(10)**	3 **(9)**	1 **(3)**
Streptococcus parasanguis	0 **(0)**	1 **(3)**	0 **(0)**
Streptococcus dysgalactiae	1 **(5)**	0 **(0)**	0 **(0)**
Streptococcus infantis	0 **(0)**	1 **(3)**	0 **(0)**
Streptococcus peroris	1 **(5)**	0 **(0)**	0 **(0)**
Streptococcus vestibularis	1 **(5)**	0 **(0)**	1 **(3)**
Unidentified streptococci	3 **(14)**	5 **(15)**	6 **(18)**
Coagulase-negative staphylococci	0 **(0)**	2 **(6)**	2 **(6)**
Rothia dentocariosa	0 **(0)**	2 **(6)**	0 **(0)**
Neisseria species	0 **(0)**	1 **(3)**	1 **(3)**
Total streptococci	21 **(100)**	29 **(85)**	30 **(91)**
Total	21	34	33

Table 5.10: Identity of Mercury-resistant Bacteria from the pre- and post-amalgam Patients

Organism	No. isolates								
	Pre Amalgam						Post Amalgam		
	Visit 1			Visit 2			Visit 3		
	Mercury concentration (μM)								
	32	64	128	32	64	128	32	64	128
Streptococcus oralis	**5**	**1**	0	**7**	0	0	**10**	**5**	0
Streptococcus mitis	**4**	**3**	0	**8**	**1**	0	**6**	0	0
Streptococcus salivarius	-	-	-	**3**	0	0	**1**	0	0
Streptococcus sanguis	**2**	0	0	**3**	0	0	**1**	0	0
Streptococcus parasanguis	-	-	-	**1**	0	0	-	-	-
Streptococcus dysgalactiae	**1**	0	0	-	-	-	-	-	-
Streptococcus infantis	-	-	-	**1**	0	0	-	-	-
Streptococcus peroris	**1**	0	0	-	-	-	-	-	-
Streptococcus vestibularis	**1**	0	0	-	-	-	**1**	0	0
Unidentified streptococci	**2**	**1**	0	**5**	0	0	**4**	**2**	0
Coagulase-negative staphylococci	-	-	-	**2**	0	0	**1**	0	**1**
Rothia dentocariosa	-	-	-	**2**	0	0	-	-	-
Neisseria species	-	-	-	**1**	0	0	**1**	0	0
Total	**16**	**5**	0	**33**	**1**	0	**25**	**7**	**1**

Table 5.11: Identity and Minimum Inhibitory Concentration ($HgCl_2$) of the Mercury-resistant Isolates

	No. (%) of Hg-resistant isolates exhibiting resistance		
	Pre-amalgam		Post-amalgam
	Visit 1	Visit 2	Visit 3
Penicillin	1 (5)	2 (6)	2 (6)
Ampicillin	0 (0)	0 (0)	0 (0)
Erythromycin	6 (29)	6 (18)	10 (30)
Vancomycin	0 (0)	1 (3)	1 (3)
Tetracycline	1 (5)	4 (12)	4 (12)
At least one antibiotic	8 (38)	7 (21)	13 (40)
One antibiotic only	8 (38)	3 (9)	11 (33)
Two antibiotics	0 (0)	3 (9)	1 (3)
Three antibiotics	0 (0)	0 (0)	0 (0)
Four antibiotics	0 (0)	1 (3)	1 (3)

Table 5.12: Resistance to Antibiotics of Mercury-resistant Bacterial Isolates from Children pre- and post-amalgam

	Number of isolates displaying resistance											
	One antibiotic			Two antibiotics			Three antibiotics			Four antibiotics		
	-Am		+Am	-Am		+Am	-Am		+Am	-Am		+Am
Mercury-resistant isolate	Visit 1	Visit 2	Visit 3	Visit1	Visit2	Visit 3	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3
S. oralis	**2**	**1**	**4**	0	0	0	0	0	0	0	0	0
S. mitis	**2**	0	**2**	0	0	0	0	0	0	0	0	0
S. salivarius	-	**1**	**1**	-	**2**	0	-	0	0	-	0	0
S. sanguis	**1**	0	0	0	0	0	0	0	0	0	0	0
S. parasanguis	-	**1**	-	-	0	-	-	0	-	-	0	-
S. dysgalactiae	0	-	-	0	-	-	0	-	-	0	-	-
S. infantis	-	0	-	-	0	-	-	0	-	-	0	-
S. peroris	0	-	-	0	-	-	0	-	-	0	-	-
S. vestibularis	**1**	-	**1**	0	-	0	0	-	0	0	-	0
Unidentified streptococci	**2**	0	**2**	0	**1**	0	0	0	0	0	0	0
CNS	-	0	**1**	-	0	**1**	-	0	0	-	0	0
Rothia dentocariosa	-	0	-	-	0	-	-	0	-	-	0	-
Neisseria species	-	0	0	-	0	0	-	0	0	-	**1**	**1**

Table 5.13: Antibiotic Resistance Profiles of Mercury-resistant Bacteria from Children pre- (-Am) and post-amalgam (+Am) Fillings

	Number of isolates displaying resistance[a]											
	Penicillin			Erythromycin			Tetracycline			Vancomycin		
	-Am		+Am	-Am		+Am	-Am		+Am	-Am		+Am
Mercury-resistant isolate	Visit1	Visit2	Visit3	Visit1	Visit2	Visit 3	Visit1	Visit2	Visit3	Visit 1	Visit 2	Visit 3
S. oralis	0	0	0	**2**	**1**	**3**	0	0	**1**	0	0	0
S. mitis	0	0	0	**1**	0	**2**	**1**	0	0	0	0	0
S. salivarius	-	**1**	0	-	**2**	**1**	-	0	0	-	0	0
S. sanguis	**1**	0	0	0	0	0	0	0	0	0	0	0
S. parasanguis	-	0	-	-	0	-	-	**1**	-	-	0	-
S. dysgalactiae	0	-	-	0	-	-	0	-	-	0	-	-
S. infantis	-	0	-	-	0	-	-	0	-	-	0	-
S. peroris	0	-	-	0	-	-	0	-	-	0	-	-
S. vestibularis	0	-	0	**1**	-	**1**	0	-	0	0	-	0
Unidentified streptococci	0	0	0	**2**	**1**	**1**	0	**1**	**1**	0	0	0
CNS	-	0	**1**	-	0	**1**	-	0	**1**	-	0	0
Rothia dentocariosa	-	0	-	-	0	-	-	0	-	-	0	-
Neisseria species	-	**1**	**1**	-	**1**	**1**	-	**1**	**1**	-	**1**	**1**

Table 5.14: Antibiotic Resistance Profiles of Mercury-resistant Bacteria from Children pre- (-Am) and post-amalgam (+Am) Fillings

[a] No ampicillin-resistant bacteria were found.

Antimicrobial agent	All streptococci (n=21)			
	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R
Mercury	32-64	32	64	100
Penicillin	≤0.008-0.5	0.03125	0.125	5
Ampicillin	≤0.008-0.5	0.0625	0.125	0
Erythromycin	0.016-4	0.03125	2	29
Vancomycin	0.25-1	0.5	1	0
Tetracycline	0.125-2	0.25	0.5	5

Table 5.15: *In vitro* activities of 5 Antimicrobial Agents against Mercury-resistant Oral Streptococci isolated from pre-amalgam Patients (Visit 1)

Antimicrobial agent	All streptococci (n=29)			
	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R
Mercury	32-64	32	32	100
Penicillin	≤0.008-1	0.03125	0.125	3
Ampicillin	≤0.008-0.25	0.0625	0.125	0
Erythromycin	0.016-8	0.03125	2	21
Vancomycin	0.125-2	0.5	1	0
Tetracycline	0.03125-16	0.25	4	10

Table 5.16: *In vitro* activities of 5 Antimicrobial Agents against Mercury-resistant Oral Streptococci isolated from pre-amalgam Patients (Visit 2)

Antimicrobial agent	All streptococci (n=30)			
	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R
Mercury	32-64	32	64	100
Penicillin	≤0.008-0.125	0.0625	0.0625	0
Ampicillin	≤0.008-0.25	0.0625	0.125	0
Erythromycin	0.016-2	0.03125	2	27
Vancomycin	0.25-1	0.5	1	0
Tetracycline	0.125-64	0.25	0.5	7

Table 5.17: *In vitro* activities of 5 Antimicrobial Agents against Mercury-resistant Oral Streptococci isolated from post-amalgam Patients (Visit 3)

Antimicrobial agent	*S. oralis* (n=6)				*S. mitis* (n=7)				Other *Streptococcus* species (n=8)			
	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R
Mercury	32-64	32	64	100	32-64	32	64	100	32-64	32	64	100
Penicillin	≤0.008-0.0625	0.03125	0.0625	0	0.016-0.125	0.0625	0.125	0	0.016-0.5	0.03125	1	12.5
Ampicillin	≤0.008-0.125	0.0625	0.125	0	0.016-0.5	0.125	0.5	0	≤0.008-0.5	0.0625	1	0
Erythromycin	0.016-1	0.03125	1	33	0.016-1	0.03125	1	14	0.016-4	0.25	4	38
Vancomycin	0.25-0.5	0.5	0.5	0	0.5	0.5	0.5	0	0.5-1	1	1	0
Tetracycline	0.125-0.5	0.25	0.5	0	0.25-2	0.25	2	14	0.125-0.5	0.25	0.5	0

Table 5.18: *In vitro* activities of 5 Antimicrobial Agents against Mercury-resistant Oral Streptococci isolated from pre-amalgam Patients (Visit 1)

Antimicrobial agent	*S. oralis* (n=7)				*S. mitis* (n=9)				Other *Streptococcus* species (n=13)			
	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R
Mercury	32	32	32	100	32-64	32	64	100	32	32	32	100
Penicillin	≤0.008-0.0625	0.03125	0.0625	0	0.03125-0.125	0.0625	0.125	0	≤0.008-1	0.03125	0.125	8
Ampicillin	≤0.008-0.125	0.0625	0.125	0	≤0.008-0.125	0.0625	0.125	0	≤0.008-0.25	0.0625	0.25	0
Erythromycin	0.016-0.25	0.03125	1	14	0.016-0.5	0.3125	1	11	0.016->8	0.03125	2	23
Vancomycin	0.25-1	0.5	1	0	0.25-1	0.5	1	0	0.5-2	1	1	0
Tetracycline	0.125-0.5	0.25	0.5	0	0.125-0.5	0.25	0.5	0	0.0625-16	0.5	8	23

Table 5.19: *In vitro* activities of 5 Antimicrobial Agents against Mercury-resistant Oral Streptococci isolated from pre-amalgam Patients (Visit 2)

Antimicrobial agent	*S. oralis* (n=15)				*S. mitis* (n=6)				Other *Streptococcus* species (n=9)			
	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R	Range	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	%R
Mercury	32-64	32	64	100	32	32	32	100	32-64	32	64	100
Penicillin	≤0.008-0.125	0.03125	0.125	0	≤0.008-0.125	0.0625	0.125	0	≤0.008-0.0625	0.0625	0.0625	0
Ampicillin	≤0.008-0.25	0.0625	0.125	0	≤0.008-0.125	0.125	0.125	0	≤0.008-0.25	0.0625	0.25	0
Erythromycin	0.016-2	0.03125	1	20	0.03125-1	0.0625	1	33	0.016-2	0.03125	2	33
Vancomycin	0.25-1	0.5	0.5	0	0.25-1	0.5	1	0	0.5-1	0.5	1	0
Tetracycline	0.125-64	0.25	0.5	7	0.25-0.5	0.25	0.5	0	0.25-32	0.5	32	11

Table 5.20: *In vitro* activities of 5 Antimicrobial Agents against Mercury-resistant Oral Streptococci isolated from post-amalgam Patients (Visit 3)

Chapter Six

Mercury Reductase (*merA*) Genes in the Oral Flora

6.0 Mercury Reductase (*merA*) Genes in the Oral Flora

6.1 Introduction

Mercury resistance is ubiquitous and has been observed in a wide variety of bacterial genera isolated from human, animal and environmental samples (Moore, 1960, Nakahara *et al*, 1977a, Porter *et al*, 1982, Khor and Jegathesan, 1983, Rudrik *et al*, 1985, Avila-Campos *et al*, 1989, Lyttle and Bowden, 1993a). The most widely reported and studied mechanism of mercury resistance is the enzymatic reduction of divalent mercuric ions (Hg^{2+}) to the metallic form (Hg^0) by the cytoplasmic flavoenzyme mercuric reductase (MerA) (Hart *et al*, 1998). The gene for this enzyme is found on the *mer* operon, among other genes required for regulation and transport and the vast majority of information has been obtained from studies of Gram-negative bacteria (Silver and Phung, 1996, Osborn *et al*, 1997). The *mer* determinants from these bacteria are often located on plasmids and/or transposons, indicating the potential for high mobility of these elements (Hobman and Brown, 1997). In comparison, less research has been undertaken on Gram-positive bacteria. The first Gram-positive mercury-resistant determinants to have been sequenced were the plasmidal *mer* operon of *Staph. aureus* pI258 and the chromosomal operons of *B. cereus* RC607 and *Streptomyces lividans* 1326 (Laddaga *et al*, 1987, Wang *et al*, 1989, Sedlmeier and Altenbuchner, 1992). Although all 3 *mer* operons contained *merA* genes homologous to Gram-negative *merA* genes, the mercuric ion transport genes showed little similarity to Gram-negative transport genes. In recent years, the *mer* operon also has been found on transposons found

in *Bacillus megaterium, B. cereus* and an *Exiguobacterium* species (Huang *et al*, 1999, Bogdanova *et al*, 2001).

The *merA* gene is long with 631 codons (1896bp). The MerA protein (69 kilodaltons) is composed of two monomers, producing a rough globular dimer (Silver and Phung, 1996). The structure of the MerA protein of *B. cereus* RC607 was established though X-ray crystallography and it is used as the model for all mercury reductases from both Gram-positive and Gram-negative bacteria (Schiering *et al*, 1991). This structure is also seen in the other members of the pyridine nucleotide-disulphide oxidoreducatase family such as glutathione reductase, lipoamide dehydrogenase and thioredoxin (Petsko, 1991). These enzymes are FAD-containing, NAD(P)H-dependent oxidoreductases that contain a redox-active disulphide in the active site. The three-dimensional structure of MerA from *B. cereus* RC607 can be divided into 3 parts (Figure 6.1). These include two N-terminal regions (residues 1-166), the core (residues 167-616) and the C-terminal extension (residues 617-631). Mercury reductase is the only member of the oxidoreductase family to possess the N-terminal region. The N-terminal domain is also absent or shorter in mercury reductases from Gram-positive bacteria with a high genomic G+C content such as *Arthrobacter*, *Citreobacterium*, *Micrococcus*, *Mycobacterium*, *Rhodococcus* and *Streptomyces* (Silver and Misra, 1988, Bogdanova and Mindlin, 1991). The N-terminal domain is easily removed by proteolysis and contains four cysteines. It was once believed to play a role in acquiring Hg^{2+} from the MerP transport protein as approximately 70 amino acids of the N-terminal domain are homologous in sequence to this protein. However, further work has concluded that the terminal acts as an

intracellular buffer, preventing inhibitory binding of other proteins in the cell to Hg^{2+} and to act as the specific delivery agent of Hg^{2+} to the catalytic core (Barkay *et al*, 2003). The presence and number of repeats of the N-terminal domain may be correlated with the type and quantity of thiol synthesised by the species. Bacteria that lack glutathione and have low intracellular concentrations of other thiols, such as members of the *Bacillus* and *Clostridium* genera (low G+C content) have double repeats in the N-terminal domain. Cells that synthesise glutathione or have high intracellular concentrations of thiols such as cysteine and coenzyme A have a single repeat in the N-terminal domain. Streptomycetes completely lack the N-terminal domain. However, there are some exceptions. Some *Bacillus* strains, which have a low genomic G+C content only have a single N-terminal repeat (Bogdanova *et al*, 1998, Narita *et al*, 2003).

The C-terminal and core region of the MerA protein are more conserved than the N-terminal domain. The C-terminal contains two cysteines, which assist in Hg^{2+} binding at the active site. Mutagenesis studies have shown that they are essential for enzyme activity (Barkay *et al*, 2003). Wang showed that in RC607, pI258, Tn501 (*P. aeruginosa*) and Tn21 (*S. flexneri*), 22 of 28 amino acids are completely conserved (Wang *et al*, 1989). The C-terminal of RC607 and pI258 are 100% identical (Wang *et al*, 1989). Specific areas of the core are conserved, such as the FAD domain, NADPH binding site and a redox-active disulphide site. In Tn21, Tn501 and pI258 these sites have been shown to be 90% identical (Laddaga *et al*, 1987).

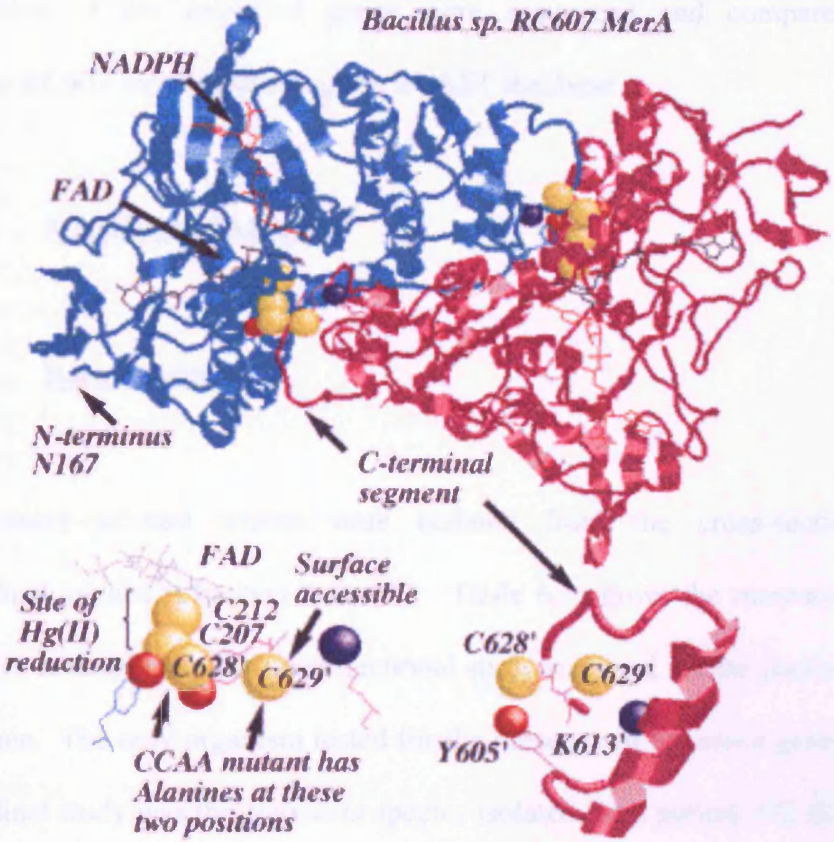

Figure 6.1: Model of MerA from Crystal Structure of *Bacillus* Enzyme

The conserved cysteine and tyrosine residues are shaded in

yellow and red respectively (Barkay *et al*, 2003)

6.2 Aims

Despite many research groups having isolated mercury-resistant bacteria from plaque and saliva samples, to date none have published information as to whether these bacteria possess the *merA* gene. The aim of this part of the study was to determine whether the mercury-resistant bacteria isolated from the cross-sectional and longitudinal studies (Chapters 4 and 5) possessed the *merA* gene. The

presence of the *merA* gene was determined by the polymerase chain reaction. A selection of the amplified genes were sequenced and compared to the *B. cereus* RC607 *merA* gene using the BLAST database.

6.3 Materials and Methods

6.3.1 Bacterial strains

The mercury-resistant strains were isolated from the cross-sectional and longitudinal studies (Chapters 4 and 5). Table 6.1 shows the mercury-resistant organisms isolated from the cross-sectional study screened for the presence of the *merA* gene. The only organism tested for the presence of the *merA* gene from the longitudinal study was the *Neisseria* species isolated from patient 332 during visit 2 (pre-amalgam) and visit 3 (post-amalgam).

Organism	No. of isolates	
	without amalgam fillings	with amalgam fillings
Streptococcus oralis	17	35
Streptococcus mitis	8	7
Streptococcus salivarius	8	5
Streptococcus sanguis	1	3
Streptococcus parasanguis	6	2
Streptococcus anginosus	2	0
Streptococcus vestibularis	1	0
Unidentified streptococci	13	10
Staphylococcus aureus	1	0
Coagulase-negative staphylococci	3	1
Rothia dentocariosa	1	5
Pseudomonas stutzeri	0	1
Total	61	69

Table 6.1: Mercury-resistant bacteria isolated from the cross-sectional study tested for the *merA* gene

The bacteria were grown overnight aerobically at 37°C in 10ml Brain Heart Infusion broth (Oxoid) in the presence of 15μM $HgCl_2$ (Sigma-Aldrich).

6.3.2 Isolation of Genomic DNA

The overnight cultures were centrifuged in a MSE Centaur 2 (MSE Scientific Instruments, Crawley, Sussex, UK) at 1000g for 3 minutes and the supernatants removed using a Pasteur pipette. The genomic DNA was isolated using Puregene™ DNA Isolation Kit (Gentra Systems, Minneapolis, USA).

6.3.3 Oligonucleotide Primers

Two sets of primers were designed using the Sigma-Genosys DNA calculator found on the company's website (www.sigma-genosys.com/order_DNAcalc.asp).

6.3.3.1 BAMERA Primers

The BAMERA primers were capable of amplifying the *merA* gene from *B. cereus* RC607 (GenBank accession AB036431)(Wang *et al*, 1989):

BAMERA-F	5'-CATCATCGGTTCTGGTGGAG-3'
BAMERA-R	5'-AGTTGTCCTAATTCCATGCC-3'

The BAMERA primers produced an amplicon 532 base pairs in length when used on the positive control *B. cereus* RC607. These primers were designed to amplify the core region of the *merA* gene (Figure 6.2).

6.3.3.2 MerA1 Primers

The second primers were designed to amplify the N-terminal domain and core region of the *merA* gene (Figure 6.2). The primer is able to amplify most known *merA* genes found in Gram-positive organisms as determined by the known *merA* sequences on the BLAST database.

MerA1-F 3'-CTGGTTGTGAAGAACAT-5'

MerA1-R 3'-TCCTTCTGCCATTGTT -5'

The MerA1 primer produced an amplicon 1246 base pairs in length when used on *B. cereus* RC607, which acted as a positive control.

6.3.4 PCR Amplification

The PCR was performed in 0.5ml eppendorf tubes (ABgene, Epsom, Surrey, UK) in a thermocycler (MWG Biotech (UK) Ltd, Milton Keynes, Bedfordshire, UK).

6.3.4.1 BAMERA Amplification

The PCR reaction mixtures were composed of 2µl DNA, 5µl 10X ThermoPol Reaction Buffer, 1U of Vent$_R$® DNA Polymerase (New England Biolabs (UK) Ltd, Hitchin, Hertfordshire), 25pmol of each primer and 10µM of deoxynucleoside triphosphates (dNTPs) (Roche Diagnostics Ltd, Lewes, East Sussex, UK). DD-H_2O was added to make a total volume of 50µl. The PCR

mixtures were denatured for 4 minutes at 94°C and then subjected to 35 cycles of amplification (30 seconds of annealing at 94°C, 30 seconds of elongation at 55°C and 1 minute of elongation at 72°C). Finally, the samples were denatured at 72°C for 5 minutes and held indefinitely at 4°C.

6.3.4.2 MerA1 Amplification

Two microlitres of the DNA template was added to a reaction mixture (50µl final volume containing 10X PCR buffer, 1.5mM $MgCl_2$, 1.25U of *Taq* DNA polymerase (Promega, Southampton, Hampshire, UK), 20pmol of each primer and 20µM of deoxynucleoside triphosphates (dNTPs) (Roche Diagnostics Ltd, Lewes, East Sussex, UK). The reactions were made up to a total volume of 50µl with sterile DD-H_2O. The PCR mixtures were denatured for 3 minutes at 94°C and then subjected to 30 cycles of amplification (1 minute of annealing at 94°C, 1 minute of elongation at 50°C and 1 minute of elongation at 72°C). Finally, the samples were denatured at 72°C for 3 minutes and held indefinitely at 4°C.

6.3.5 Sequencing the Amplified Products

The PCR products were cleaned-up using the Qiagen QIAquick® kit (Qiagen Ltd, Crawley, West Sussex, UK) and the concentration determined by reading the OD at 260nm using a spectrophotometer (Pharmacia Biotech). PCR products in low concentrations were used neat, while highly concentrated PCR products were diluted with DD-H_2O. Products produced using the BAMERA and MerA1 primers were sequenced overnight using a single BAMERA and MerA1 primer

respectively. This is described in further detail in Chapter 2.2.4. The method used to clean-up the products for sequencing and the sequencing technique is described further in Chapter 2.2.4.

6.4 Results

A total of 130 organisms isolated from the cross-sectional study and the 2 *Neisseria* strains isolated from the longitudinal study were screened for the *merA* gene using the BAMERA and MerA1 primers. The MerA1 primers produced amplicons of approximately 1246 base pairs and the BAMERA primers produced products of 532 base pairs. Isolates that produced amplicons of approximately these lengths were presumed to contain the *merA* gene (Table 6.2).

Of the 52 mercury-resistant *S. oralis* tested, 46 (88%) contained the *merA* gene, while of the 15 *S. mitis* tested, 9 of the organisms contained the gene (60%). The only streptococcus species found to not contain the *merA* gene was *S. sanguinis* and *S. vestibularis*. However, very few isolates of these species were tested (4 and 1 respectively).

The mercury-resistant *Staph. aureus* isolated from a patient without amalgam was found to not contain the *merA* gene when screened with the BAMERA and MerA1 primers. However, of the 4 mercury-resistant CNS tested, 2 contained the *merA* gene. One strain, which was unidentifiable and isolated from a patient without amalgam, was positive with both primers. The *Staph. epidermidis*, isolated from a patient with amalgam, was positive with the MERA1 primer only.

One *R. dentocariosa* strain, isolated from a patient with amalgam, was found to contain a *merA* gene when tested with the both the BAMERA and MerA1 primers.

The *P. stutzeri*, isolated from a patient with amalgam fillings, did not contain the *merA* gene when tested with both sets of primers.

The 2 *N. sicca* isolates, which were isolated during the longitudinal study from a patient pre- and post-amalgam, were found to contain the *merA* gene when tested with both the BAMERA and MerA1 primers.

Of the two primers, the BAMERA primer produced more amplicons and thus detected more strains with *merA* than the MerA1 primer (Table 6.3 & 6.4). Of the 52 *S. oralis* tested, 81% were *merA* positive when screened with the BAMERA primers, but only 42% were found to possess the *merA* when tested using the MerA1 primers.

One quarter of the amplified products were sequenced and compared to the *B. cereus* RC607 *merA* gene using the BLAST database (www.ncbi.nlm.nih.gov/BLAST). All sequenced amplicons were found to be up to 95% identical to the *B. cereus* RC607 *merA* gene. The amplicons were also found to be very similar to *merA* genes from *B. megaterium*, *B. macroides*, *B. licheniformis*, *B. sphaericus*, *Exiguobacterium* species and *Clostridium butyricum*.

6.5 Discussion

Despite mercury resistance being discovered first in *Staph. aureus*, less is known about the *mer* operons of Gram-positive bacteria than Gram-negative bacteria (Richmond & John, 1964). The work that has been published has focussed on environmental strains such as *B. cereus* (Wang *et al*, 1989, Bogdanova *et al*, 1998), *B. megaterium* (Narita *et al*, 2003), *Exiguobacterium* (Nikiforov *et al*, 1999) and *Streptomyces* (Sedlmeier and Altenbuchner, 1992). To date, there is little published information about the *mer* operon of Gram-positive clinical isolates and there is no information in publications or the BLAST database about the *mer* operon in viridans streptococci and commensal *Neisseria* strains (Laddaga *et al*, 1987, Zscheck and Murray, 1990). Therefore, the aim of this study was to determine whether Hg^R bacteria isolated from saliva and plaque samples possessed the *merA* gene and, if positive, a selection of these genes were sequenced and compared to others on the BLAST database.

Of the mercury-resistant organisms isolated, members of the genera *Staphylococcus*, *Streptococcus*, *Rothia* and *Neisseria* were found to contain the *merA* gene. The *merA* gene was not present in the *P. stutzeri*.

The BAMERA primers determined that 53% of the organisms tested contained the *merA* gene, while only 33% of the strains contained the *merA* gene when screened with the MerA1 primers. However, it is possible that the PCR negative strains contained the gene and the primers used were unable to amplify the *merA* gene. The BAMERA primers amplified only the core region of the *merA* gene,

producing an amplicon of 532bp in length. This area included the conserved regions of Cys207, Cys212 and Tyr264. The MerA1 primers were designed to amplify the second repeat of the N-terminal domain and the core region of the RC607 *merA* gene, producing an amplicon of 1246bp. This area includes the conserved regions of Cys207, Cys212, Tyr265 and Tyr605 and a single N-terminal cysteine, which is involved in delivery of Hg^{2+} to the catalytic core. Of the 113 streptococci tested, 65 (58%) were positive for the *merA* gene when screened with the BAMERA primers, while only 39 (35%) possessed the gene when tested with the MerA1 primers. This suggests that the streptococci that were negative with the MerA1 primer but positive with the BAMERA primer do not possess an N-terminal domain. However, taxonomically, streptococci, like the genus *Clostridium* and *Bacillus*, have a low G+C content. Previous work has shown that Gram-positive bacteria with a genomic G+C content less than 50% possess a N-terminal domain (Bogdanova and Mindlin, 1991, Narita *et al*, 2003). Therefore, according to this rule, streptococci should contain an N-terminal repeat and the MerA1 primer should amplify just as many genes as the BAMERA primer when used to amplify the *merA* gene in streptococci. It is possible that the streptococci tested do have an N-terminal domain but the nucleotides found within it differ significantly to RC607 and thus the MerA1 primer is simply unable to amplify the streptococcal *merA* gene.

Of the 5 staphylococci tested (1 *Staph. aureus* and 4 CNS), only 2 were found to contain the *merA* gene. Only one of these strains appeared positive with the MerA1 primer suggesting it has an N-terminal domain. Staphylococci have a low G+C content and high concentrations of cysteine and coenzyme A in the

cytoplasm (Barkay *et al*, 2003). This suggests that the *merA* should possess an N-terminal domain. In fact, the mercuric reductase from *Staph. aureus* pI258 contains a single repeat in the N-terminal domain (Laddaga *et al*, 1987).

Only one of the 6 *R. dentocariosa* strains isolated was found to contain the *merA* gene. This was positive using the MerA1 primer, suggesting that the *merA* gene from this organism has an N-terminal domain. *Rothia* belong to the G+C rich branch of the Gram-positive bacteria and this may indicate that, like other bacteria with a high G+C content, the *merA* gene contains a single repeat at the N-terminal domain.

Three Gram-negative mercury-resistant bacteria were isolated from patients in the cross-sectional (*P. stutzeri*) and longitudinal (*N. sicca*) studies. Using PCR, the *P. stutzeri* was found to not contain the *merA* gene. However, the organism may possess *merA* but the homology between the gene and primers may be low and unable to amplify. Barkay isolated an environmental Hg^R *P. stutzeri* that hybridised with a *merA* probe at a high stringency (Barkay *et al*, 2003). The probe was developed from the *merA* gene of Tn501. The investigation did not determine whether the strains possessed a *merA* gene with an N-terminal domain. However, other studies have determined that pseudomonads synthesise glutathione, which suggests that they have a single repeat in the MerA appendages (Barkay *et al*, 2003). The two *Neisseria* isolates from a patient enrolled in the longitudinal study were found to contain the *merA* gene when screened using both the MerA1 and BAMERA primers. This suggests that the gene has an N-terminal domain. At present, no information has been published on mercury resistance and

merA in *Neisseria* species, although other Gram-negative bacteria usually possess a single repeat in the N-terminal domain (Wang *et al*, 1989, Narita *et al*, 2003). The amplicons were sequenced and found to be up to 95% identical to the *Bacillus*, *Exiguobacterium* and *Clostridium* sequences on the BLAST database.

Amplicons from some of the streptococci, both *Neisseria* and one of the staphylococci were sequenced to determine their similarity to the RC607 *merA* gene. The amplicons from the *R. dentocariosa* were not sequenced. The amplicons that were sequenced were found to be identical to, or very closely related to, the *merA* gene of *B. cereus* RC607.

Of the organisms tested, 28% of the organisms from the patients with amalgam and 52% of the bacteria from the amalgam-free patients were found to not contain the *merA* gene when screened using PCR. These strains may either contain a gene significantly divergent from the *merA* gene from *B. cereus* RC607 to preclude amplification or may not possess mercury reductase and have a different resistance system. Using a less specific technique such as low-stringent hybridisation may have resulted in detecting the *merA* gene in more of the organisms tested. Hybridisation has been used in many studies where probes encoding fragments of *merA* from pI258 has detected the gene in *Ent. faecalis* and probes encoding fragments of Tn501 detected the gene in *P. stutzeri*, *P. cepacia* and *P. vesicularis* (Zscheck and Murray, 1990, Barkay *et al*, 2003). Low stringency hybridisation has permitted hybrid formation between the *merA* genes from Tn501, RC607 and pI258 (Barkay *et al*, 2003).

In previous studies, many phenotypic mercury-sensitive bacteria have been shown to be genotypically mercury-resistant (Bogdanova *et al*, 1992, Fleischmann *et al*, 1995). A collection of Gram-positive bacteria isolated from environmental samples were shown to possess *merR* and *merA* genes but were phenotypically mercury-sensitive (Bogdanova *et al*, 1992). Bogdanova concluded this was probably due to the absence or inactivation of genes encoding transport functions. Such vestigual or cryptic operons have also been detected in *Haemophilus influenzae* where the genes for transport and regulation were separated by over 600kb of DNA (Fleischmann *et al*, 1995). Cryptic operons have also been found in *Staphylococcus* species and Gram-negative bacteria. *Micrococcus*, *Brevibacterium* and *Rhodococcus* strains (G+C rich bacteria) also possess cryptic *mer* determinants (Summers *et al*, 1986, Bogdanova *et al*, 1992, Silver and Walderhaug, 1992).

Due to time constraints, this part of the study was not completely finished and many questions have been left unanswered. Previous work has shown that most Gram-positive bacteria exhibit broad-spectrum resistance to mercury and this is especially common in bacteria with double repeats in their N-terminal domain (Bogdanova *et al*, 1998, Narita *et al*, 2003). The length of the N-terminal domain can be determined by the decrease of the molecular mass of mercury reductase in limited proteolysis experiments (Bogdanova and Mindlin, 1991). To continue the present study, testing for growth on agar containing an organomercurial would establish whether the mercury-resistant bacteria had broad-spectrum resistance. The isolates could be tested using PCR with different primers, which would determine the type of N-terminal domain that the organisms contain.

Alternatively, proteolysis studies would determine whether the bacteria had single or double repeats at the N-terminal domain. Further work could also involve the determination of whether the *mer* operons are found on the chromosome or a plasmid.

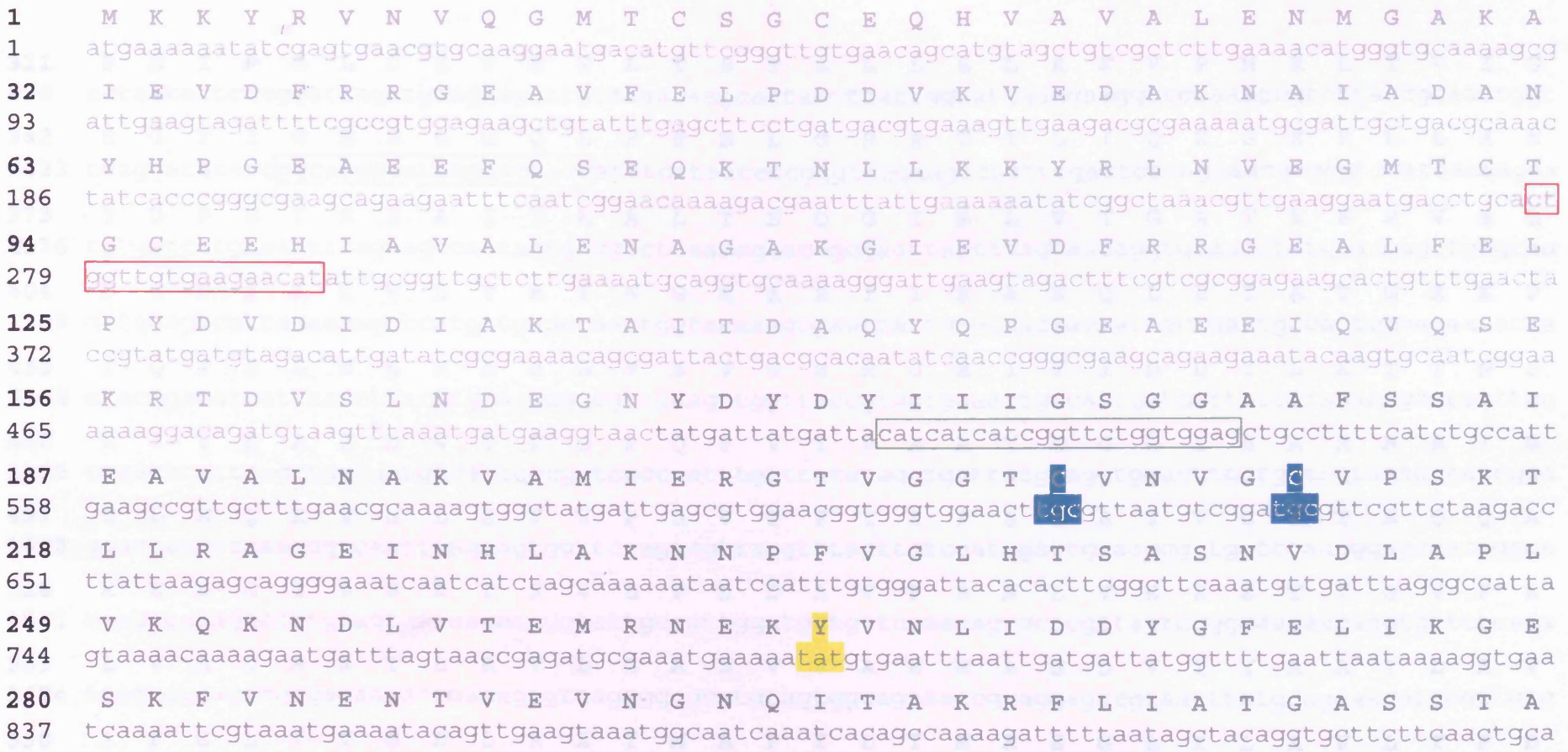

Figure 6.2: Nucleotide **(amino acid)** sequence of *merA* **(MerA)** *B. cereus* RC607

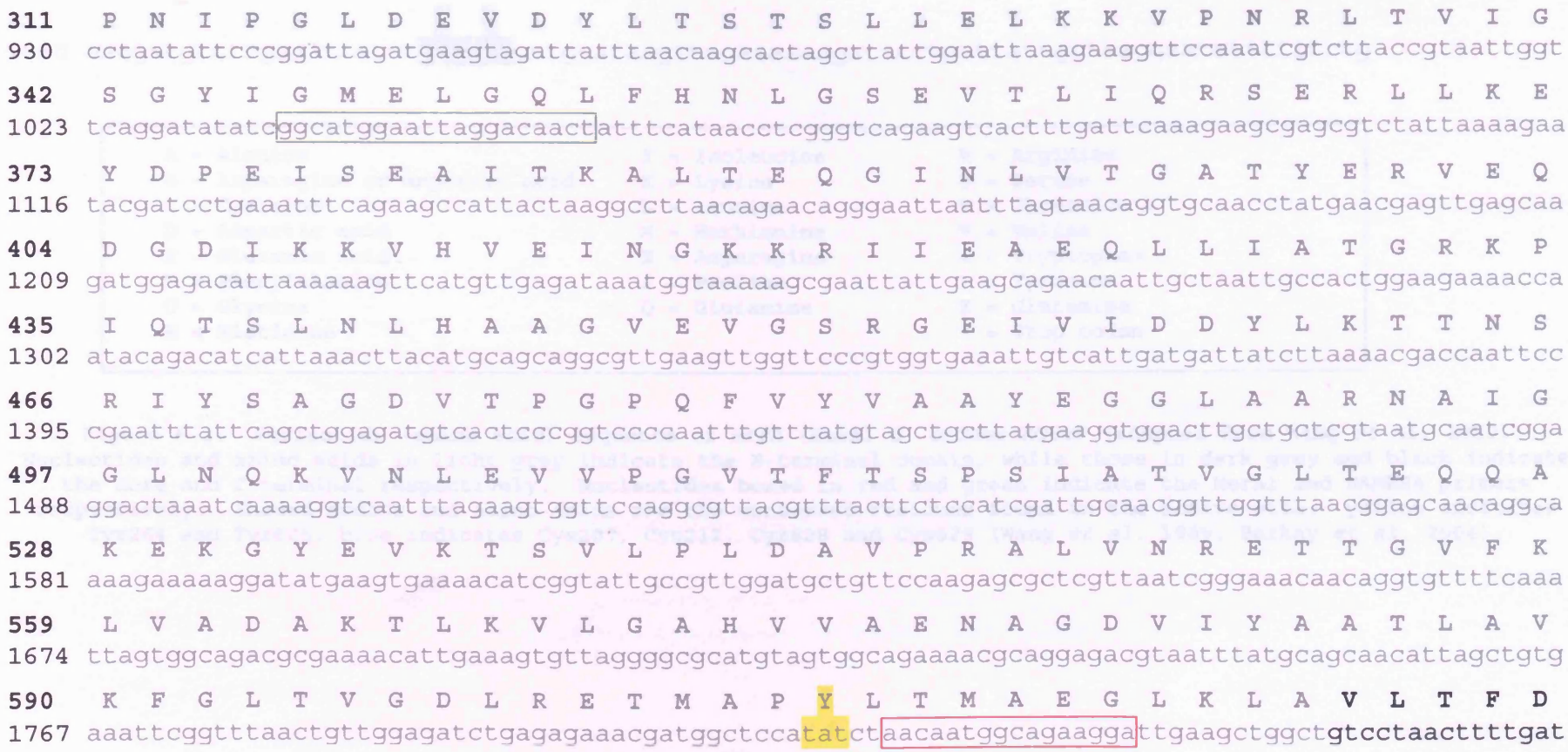

Figure 6.2 continued: Nucleotide **(amino acid)** sequence of *merA* **(MerA)** *B. cereus* RC607

621 K D V S K L S C C A G * V R K K I P D Y C S G I F F L

1860 aaagatgtttcgaaattatcttgctgtgctggataagttagaaaaaagattcctgattattgctcaggaatctttttctg

A = Alanine	I = Isoleucine	R = Arginine
B = Asparagine or Aspartic acid	K = Lysine	S = Serine
C = Cysteine	L = Leucine	T = Threonine
D = Aspartic acid	M = Methionine	V = Valine
E = Glutamic acid	N = Asparagine	W = Tryptophan
F = Phenylalanine	P = Proline	Y = Tyrosine
G = Glycine	Q = Glutamine	Z = Glutamine
H = Histidine		* = Stop codon

Figure 6.2: Nucleotide **(amino acid)** sequence of *merA* **(MerA)** *B. cereus* RC607 (adapted from Wang *et al*, 1989) Nucleotides and amino acids in light grey indicate the N-terminal domain, while those in dark grey and black indicate the core and C-terminal respectively. Nucleotides boxed in red and green indicate the MerA1 and BAMERA primers respectively. Shaded codons and amino acids are the conserved residues found in the active site: yellow indicates Tyr264 and Tyr605, blue indicates Cys207, Cys212, Cys628 and Cys629 (Wang *et al*, 1989, Barkay *et al*, 2004).

Organism	No. (%) isolates containing the *merA* gene	
	without amalgam fillings	with amalgam fillings
Streptococcus oralis	13 **(76)**	33 **(94)**
Streptococcus mitis	3 **(38)**	6 **(86)**
Streptococcus salivarius	2 **(25)**	2 **(40)**
Streptococcus parasanguis	1 **(17)**	1 **(50)**
Streptococcus sanguis	0 **(0)**	0 **(0)**
Streptococcus anginosus	1 **(50)**	-
Streptococcus vestibularis	0 **(0)**	-
Unidentified streptococci	8 **(62)**	6 **(60)**
Staphylococcus aureus	0 **(0)**	-
Coagulase-negative staphylococci	1 **(33)**	1 **(100)**
Rothia dentocariosa	0 **(0)**	1 **(20)**
Pseudomonas stutzeri	-	0 **(0)**
Total	29 **(48)**	50 **(72)**

Table 6.2: Number **(%)** of Mercury-resistant isolates (cross-sectional study) containing the *merA* gene as determined using the BAMERA and MerA1 primers

Organism	No. of organisms tested	No. (%) of organisms containing the *merA* gene	
		BAMERA	MerA1
Streptococcus oralis	52	42 **(81)**	22 **(42)**
Streptococcus mitis	15	8 **(53)**	5 **(33)**
Streptococcus salivarius	13	3 **(23)**	2 **(15)**
Streptococcus parasanguis	8	2 **(25)**	0 **(0)**
Streptococcus sanguis	4	0 **(0)**	0 **(0)**
Streptococcus anginosus	2	1 **(50)**	0 **(0)**
Streptococcus vestibularis	1	0 **(0)**	0 **(0)**
Unidentified streptococci	23	10 **(23)**	10 **(43)**
Staphylococcus aureus	1	0 **(0)**	0 **(0)**
Coagulase-negative staphylococci	4	1 **(25)**	2 **(50)**
Rothia dentocariosa	6	1 **(17)**	1 **(17)**
Pseudomonas stutzeri	1	0 **(0)**	0 **(0)**
Total	130	68 **(52)**	42 **(32)**

Table 6.3: Number **(%)** of mercury-resistant isolates (cross-sectional study) containing the *merA* gene as determined using the BAMERA and MerA1 primers

Organism	No. of organisms tested	No. (%) of organisms containing the *merA* gene			
		BAMERA only	MerA1 only	MerA1 and BAMERA	Neither
Streptococcus oralis	50	26 **(52)**	6 **(12)**	16 **(32)**	2 **(4)**
Streptococcus mitis	15	5 **(33)**	2 **(27)**	3 **(20)**	5 **(33)**
Streptococcus salivarius	13	2 **(15)**	1 **(8)**	1 **(8)**	9 **(69)**
Streptococcus parasanguis	7	2 **(29)**	0 **(0)**	0 **(0)**	5 **(71)**
Streptococcus sanguis	4	0 **(0)**	0 **(0)**	0 **(0)**	4 **(100)**
Streptococcus anginosus	1	0 **(0)**	0 **(0)**	0 **(0)**	1 **(100)**
Unidentified streptococci	23	4 **(17)**	4 **(17)**	6 **(4)**	9 **(39)**
Total	113	39 **(35)**	13 **(11)**	26 **(23)**	35 **(31)**

Table 6.4: Number **(%)** of mercury-resistant streptococci (cross-sectional study) containing the *merA* gene as determined using the BAMERA and MerA1 primers

Chapter Seven

Conclusions

7.0 Conclusions

7.1 Introduction

The oral flora of humans is a potential reservoir of antibiotic-resistant bacteria and studies have shown a correlation between consumption of antimicrobial agents and the emergence of resistance to these drugs. Additional factors in the environment may also give rise to selective pressure, resulting in an increase in drug resistance. Detergents containing organomercurials have been shown to promote mercury resistance. Mercury resistance genes are often found on transferable elements, which are able to carry other resistance genes, conferring resistance to other antimicrobials such as antibiotics. It is feasible that environmental mercury could promote the emergence and spread of both mercury and antibiotic resistance in the commensal flora of humans. The most prevalent source of intentional mercury exposure for the general population in developed countries is dental amalgam.

A small number of previous studies have investigated whether the dental amalgam used to repair caries can provide enough selective pressure to promote the emergence and spread of mercury and antimicrobial resistance in the normal flora of humans and monkeys (Summers *et al*, 1993, Österblad *et al*, 1995, Edlund *et al*, 1996). However, from the limited number of studies carried out, the results have been inconclusive. Summers found a link between amalgam fillings and an increase in mercury and antibiotic resistance in the oral and faecal flora of primates (Summers *et al*, 1993). By contrast, Edlund found that, in human adults,

there was no link between the presence of mercury amalgam fillings and the prevalence of mercury-resistant oral bacteria (Edlund *et al*, 1996). The results of a study by Österblad also supports the theory that amalgam fillings do not provide selective pressure (Österblad *et al*, 1995).

The primary objective of the current study was to determine whether mercury released from amalgam fillings could select for mercury-resistant bacteria in the oral flora of children. This was achieved through both a cross-sectional and a longitudinal study. The second aim was to determine whether changes in mercury resistance in the oral flora correlated with changes in the incidence of antibiotic resistance. The final aim was to determine whether individual mercury-resistant isolates contained the *merA* gene.

7.2 Effect of Medium Composition on the Susceptibility of Oral Streptococci to Mercuric Chloride

Unlike antibiotic susceptibility testing, where the MIC of the antibiotic against an organism can be determined using standardised methods, mercury susceptibility testing cannot be carried out in this manner. This has led to the use of a wide range of methods to determine whether organisms are 'resistant' to mercury.

The current study has shown that the culture medium used in mercury susceptibility testing plays a very important role and can have a significant influence on the MIC. Significant interactions between mercuric chloride and test media were observed and adding blood to solid media strongly decreased the

antibacterial activity of mercury. The study highlighted that before undertaking studies of mercury resistance it is important to choose the correct agar. Not only must the agar support the growth of the organisms tested but also its interaction with mercury must be determined, so that the correct concentration of mercury is incorporated.

7.3 A Cross-sectional Study – Resistance of the Commensal Oral Microflora to Mercury and Antibiotics in Subjects With and Without Dental Amalgam Fillings

In the cross-sectional study, samples were taken from 2 sets of patients, one with amalgam fillings and one without, to determine whether children with fillings harboured a higher proportion of mercury-resistant bacteria in their oral flora than those without. The mercury-resistant bacteria were identified and differences in the types of organism isolated from individuals with and without amalgam fillings were noted. Finally, the MIC of 6 frequently-used antibiotics for the mercury-resistant organisms were determined in order to investigate a possible correlation between antibiotic and mercury resistance.

It was observed that similar numbers of children without and with amalgam fillings harboured mercury-resistant bacteria in their oral flora and these differences were not statistically significant. However, the proportion of mercury-resistant oral bacteria as a fraction of the total sample population showed a significant correlation with the number of amalgam surfaces in the children's mouths.

There was little difference between the two groups in terms of the number of different mercury-resistant species isolated. Mercury-resistant organisms isolated included *Streptococcus* species, *Staph. aureus*, coagulase-negative staphylococci (CNS), *Rothia dentocariosa* and *Pseudomonas stutzeri*. No mercury-resistant obligate anaerobes were isolated from either group. Most of the mercury-resistant bacteria isolated were streptococci and previous studies have shown that streptococci are the predominant cultivable oral flora in children between the ages of 2 and 10 years (Chen *et al*, 1997, Kamma *et al*, 2000). Of those streptococci that could be identified, the majority were *S. oralis* in both groups. Mercury-resistant *S. oralis* was isolated more often from the amalgam group and this was found to be statistically significant. In the non-amalgam group, the mercury-resistant streptococci showed a greater species diversity with no particular streptococcal species being dominant in terms of frequency of isolation.

Forty one percent and 33% of the mercury-resistant bacteria isolated from the groups with and without amalgam fillings respectively were also resistant to at least one of the antibiotics tested. The mercury-resistant organisms were most often resistant to tetracycline and to a lesser extent, erythromycin. Ampicillin and vancomycin resistance was not observed in any of the Gram-positive organisms isolated. A greater number of *S. oralis* strains isolated from patients with amalgam were resistant to tetracycline compared with the number of *S. oralis* strains from the non-amalgam patients. These results were statistically significant.

7.4 A Longitudinal Study – Resistance of the Commensal Oral Microflora to Mercury and Antibiotics in Subjects before and after the placement of Amalgam Fillings

The first aim of the longitudinal study was to determine whether placement of mercury amalgam fillings in children's teeth resulted in an increase in oral bacteria resistant to mercury and various antibiotics. This was achieved by measuring the proportion of mercury- and antibiotic-resistant bacteria over three visits before and after amalgam placement. A comparison of the results demonstrated no significant difference in the proportion of bacteria resistant to either mercury or to any of the antibiotics between the three visits.

The second aim was to determine whether there were any differences in the types of organisms isolated from the pre- and post-amalgam samples. In general, the number of mercury-resistant species isolated did not differ greatly pre- and post-amalgam. Mercury-resistant organisms isolated included *Streptococcus* species, coagulase-negative staphylococci (CNS), *Rothia dentocariosa* and a *Neisseria* species. Ninety one percent of the organisms isolated from the children pre- and post-amalgam were identified as streptococci. *Streptococcus oralis* was the most commonly isolated streptococcal species and was more frequently isolated from post-amalgam patients. However, unlike in the cross-sectional study, this difference was narrowly not significant.

The final aim was to investigate whether the mercury-resistant organisms were also resistant to any of 5 antibiotics and to determine whether there were

differences pre- and post-amalgam placement. Twenty seven percent and 40% of the mercury-resistant bacteria isolated from the pre- and post-amalgam patients were also resistant to at least one of the antibiotics tested – however, this difference was not statistically significant. None of the mercury-resistant bacteria exhibited resistance to ampicillin or vancomycin. The mercury-resistant organisms were most often resistant to erythromycin and to a lesser extent, tetracycline. This is in sharp contrast to the cross-sectional study where more mercury-resistant isolates were resistant to tetracycline than to erythromycin.

7.5 Mercury Reductase (*merA*) Genes in Oral Flora

For the final part of this study, the polymerase chain reaction was used to determine whether the mercury-resistant bacteria isolated from the cross-sectional study and the *Neisseria* from the longitudinal study possessed the *merA* gene.

Of the mercury-resistant organisms isolated, members of the genera *Staphylococcus*, *Streptococcus*, *Rothia* and *Neisseria* were found to contain the *merA* gene. The *merA* gene was not amplified from the *P. stutzeri* as the homology between this gene and the *merA* gene from *B. cereus* RC607 may be low. Sequencing a selection of the amplicons found that they were up to 95% identical to the *Bacillus*, *Exiguobacterium* and *Clostridium* sequences on the BLAST database.

7.6 Concluding Remarks

In conclusion, mercury and antibiotic resistance in the oral flora is widely distributed among healthy children and dental amalgam alone does not appear to be a key factor in promoting spread. This study has shown that antibiotic and mercury resistance is commonly encountered in viridans streptococci which can act as genetic reservoirs. Resistance to commonly used antimicrobial agents suggests the need for comprehensive surveillance programmes to monitor resistance in these and other commensal organisms.

7.7 Parallel Work

Many viridans streptococci such as *S. mutans*, *S. mitis*, *S. oralis*, *S. sanguinis* and *S. infantis* are naturally competent (Gaustad, 1985, Ronda *et al*, 1988, Whatmore *et al*, 2000, Cvitkovitch, 2001, Ween *et al*, 2002). Additional work was carried out by a final year undergraduate where DNA was extracted from 4 mercury- and antibiotic-resistant streptococci and used in transformation experiments. However, no transformants were obtained. Mercury- and antibiotic-resistant streptococci were also used in plate and filter matings (conjugation) which failed to produce mercury-resistant transconjugants but which created tetracycline-resistant transconjugants (Stapleton *et al*, 2004).

7.8 Suggestions for Further Work

Due to time limitations, many questions remain unanswered. Previous work has shown that most Gram-positive bacteria exhibit broad-spectrum resistance to mercury and testing for growth on agar containing an organomercurial would establish whether bacteria possess broad-spectrum resistance to mercury. Broad-spectrum mercury resistance is especially common in bacteria with double repeats in the N-terminal domain of the merA protein and the length of the N-terminal can be determined by the decrease of the molecular mass of mercury reductase in proteolysis experiments (Bogdanova & Mindlin, 1991, Bogdanova *et al*, 1998, Narita *et al*, 2003). Alternatively, testing the isolates using PCR with different primers would determine the type of N-terminal that the organisms possess. It would also be interesting to determine whether the *mer* operons are found chromosomally or on a plasmid.

Many phenotypic mercury-sensitive bacteria have been shown to be genotypically mercury-resistant and possess the *merA* and *merR* gene (Bogdanova *et al*, 1992, Fleischmann *et al*, 1995). Such vestigial or cryptic operons have also been detected in *Haemophilus influenzae*, *Staphylococcus*, *Micrococcus*, *Brevibacterium* and *Rhodococcus* species and Gram-negative bacteria (Summers *et al*, 1986, Bogdanova *et al*, 1992, Silver & Walderhaug, 1992). It would be interesting to screen mercury-sensitive streptococci for the *merA* gene.

To date, very little information has been published on mercury resistance in *Neisseria*, especially *N. sicca*, which was isolated in the current study. It would be interesting to continue working further on this organism by sequencing the *merA* gene and determining whether it is plasmidal or chromosomal.

Chapter Eight

References

8.0 References

Alaluusua, S., 1991, Transmission of mutans streptococci. *Proceedings of the Finnish Dental Society*, **87**, 443-447.

Alderson, G., Ritchie, D. A., Cappellano, C., Cool, R. H., Ivanova, N. M., Huddleston, A. S., Flaxman, C. S., Kristufek, V. & A. Lounes, 1993, Physiology and genetics of antibiotic production and resistance. *Research in Microbiology*, **144**, 665-672.

Andrews, J. M., 2001a, The development of the BSAC standardized method of disc diffusion testing. *Journal of Antimicrobial Chemotherapy*, **48** (Supplement 1), 29-42.

Andrews, J. M., 2001b, Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, **48** (Supplement 1), 5-16.

Arpin, C., Canron, M. H., Maugein, J. & C. Quentin, 1999, Incidence of *mefA* and *mefE* genes in viridans group streptococci. *Antimicrobial Agents and Chemotherapy*, **43**, 2335-2336.

Arthur, M. & P. Courvalin, 1993, Genetics and mechanisms of glycopeptide resistance in enterococci. *Antimicrobial Agents and Chemotherapy*, **37**, 1563-1571.

Arzese, A. R., Tomasetig, L. & G. Botta, 2000, Detection of *tetQ* and *ermF* antibiotic resistance genes in *Prevotella* and *Porphyromonas* isolates from clinical specimens and resident microbiota of humans. *Journal of Antimicrobial Chemotherapy*, **45**, 577- 582.

Austin, D. J., Kakeshashi, M. & R. M. Anderson, 1997, The transmission dynamics of antibiotic-resistant bacteria: the relationship between resistance in commensal organisms and antibiotic consumption. *Proceedings of the Royal Society London B*, **264**, 1629-1638.

Avila-Campos, M. J., Farias, L. M., Carvalho, M. A. R., Damasceno, C. A. V., Chartone-Souza, E. & E. O. Cisaplino, 1989, *Actinobacillus (Haemophilus) actinomycetemcomitans* resistance to mercuric chloride of 41 strains isolated in Brazil. *Research in Microbiology*, **140**, 51-55.

Avila-Campos, M. J., Carvalho, M. A. R., Damasceno, C. A. V., Chartone-Souza, E. & E. O. Cisaplino, 1991a, Populational stability in species of the *Bacteroides fragilis* group under mercuric chloride action. *Revista de Microbiolgica*, **22**, 93-96.

Avila-Campos, M. J., Carvalho, M. A. R., Damasceno, C. A. V., Chartone-Souza, E. & E. O. Cisalpino, 1991b, Sensitivity to mercuric chloride of *Bacteroides fragilis* group isolates in different growth media: medium-dependent variation. *Reviews in Microbiology*, **22**, 232-236.

Bailit, H. L., Baldwin, D. C. & E. E. Hunt Jr, 1964, Increasing prevalence of gingival *Bacteroides melanogenicus* with age in children. *Archives in Oral Biology*, **9**, 435-438.

Baker, P. J., Evans, R. T., Slots, J. & R. J. Genco, 1985, Antibiotic susceptibility of anaerobic bacteria from the human oral cavity. *Journal of Dental Research*, **64**, 1233-1244.

Bantar, C., Fernandez Canigia, L., Relloso, S., Lanza, A., Bianchini, H. & J. Smayevsky, 1996, Species belonging to the '*Streptococcus milleri*' group: antimicrobial susceptibility and comparative prevalence in significant clinical specimens. *Journal of Clinical Microbiology*, **34**, 2020-2022.

Barbieri, P., Galassi, G. & E. Galli, 1989, Plasmid-encoded mercury resistance in a *Pseudomonas stutzeri* strain that degrades o-xylene. *FEMS Microbiology Ecology*, **62**, 375-384.

Barkay, T., Miller, S. M. & A. O. Summers, 2003, Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiology Reviews*, **27**, 355-384.

Barnard, J. P. & M. W. Stinson, 1999, Influence of environmental conditions on hydrogen peroxide formation by *Streptococcus gordonii*. *Infection and Immunity*, **67**, 6558-6564.

Bax, R., Mullan, N. & J. Verhoef, 2000, The millennium bugs - the need for and development of new antimicrobials. *International Journal of Antimicrobial Agents*, **16**, 51-59.

Begley, T. P., Walts, A. E. & C. T. Walsh, 1986, Mechanistic studies of protonolytic organomercurial cleaving enzyme: bacterial organomercurial lyase. *Biochemistry*, **25**, 7192-7200.

Beighton, D., J. M. Hardie and R. A. Whiley, 1991, A scheme for the identification of viridans streptococci. *Journal of Medical Microbiology*, **35**, 367-372.

Belli, W. A. & R. E. Marquis, 1991, Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. *Applied and Environmental Microbiology*, **57**, 1134-1138.

Belliveau, B. H., Starodub, M. E & J. T. Trevors, 1991, Occurrence of antibiotic and metal resistance and plasmids in *Bacillus* strains isolated from marine sediment. *Canadian Journal of Microbiology*, **37**, 513-520.

Bender, G. R., Sutton, S. V. & R. E. Marquis, 1986, Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. *Infection and Immunity*, **53**, 331-338.

Bennett, P. M., 1999, Integrons and gene cassettes: a genetic construction kit for bacteria. *Journal of Antimicrobial Chemotherapy*, **43**, 1-4.

Berg, G. G. & E. F. Miles, 1978, Binding of mercurials to membrane suspensions and undenatured proteins. *Membrane Biochemistry*, **2**, 117-134.

Berkowitz, R. J., Jordan, H. V. & G. White, 1975, The early establishment of *Streptococcus mutans* in the mouths of infants. *Archives in Oral Biology*, **20**, 171-174.

Bishara, S. E., Hoppens, B. J., Jakobsen, J. R. & F. J. Kohout, 1988, Changes in the molar relationship between the deciduous and permanent dentitions: a longitudinal study. *American Journal of Orthodontics and Dentofacial Orthopediatrics*, **93**, 19-28.

Blancou, J., 1995, History of disinfection from early times until the end of the 18th century. *Revue Scientifique et Technique de l Office International des Epizooties*, **14**, 31-39.

Bloomquist, C. G., Reilly, B. E. & W. F. Liljemark, 1996, Adherence, accumulation, and cell division of a natural adherent bacterial population. *Journal of Bacteriology*, **178**, 1172-1177.

Blumberg, P. M. & J. L. Strominger, 1974, Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. *Bacteriology Reviews*, **38**, 291-335.

Bogdanova, E. S. & S. Z. Mindlin, 1991, Occurrence of two structural types of mercury reductases among gram-positive bacteria. *FEMS Microbiology Letters*, **62**, 277-280.

Bogdanova, E. S., Mindlin, S. Z., Pakrova, E., Kocur, M. & D. A. Rouch, 1992, Mercuric reductase in environmental Gram-positive bacteria sensitive to mercury. *FEMS Microbiology Letters*, **97**, 95-100.

Bogdanova, E. S., Bass, I. A., Minakhin, L. S., Petrova, M. A., Mindlin, S. Z., Volodin, A. A., Kalyaeva, E. S., Tiedje, J. M., Hobman, J. L., Brown, N. L. & V. G. Nikiforov, 1998, Horizontal spread of mer operons among gram-positive bacteria in natural environments. *Microbiology*, **144**, 609-620.

Bogdanova, E., Minakhin, L., Bass, I., Volodin, A., Hobman, J. L. & V. Nikiforov, 2001, Class II broad-spectrum mercury resistance transposons in Gram-positive bacteria from natural environments. *Research in Microbiology*, **152**, 503-514.

Bonafede, M. & L. B. Rice, 1997, Emerging antibiotic resistance. *Journal of Laboratory Clinical Medicine*, **130**, 558-566.

Bouwsma, O. J., 1996, The status, future, and problems of oral antiseptics. *Current Opinion in Periodontology*, **3**, 78-84.

Bowden, G. H., 1969, The components of the cell wall and extracellular slime of four strains of *Staphylococcus salivarius* isolated from human dental plaque. *Archives of Oral Biology*, **14**, 685-697.

Bowden, G. H. & Y. H. Li, 1997, Nutritional influences on biofilm development. *Advances in Dental Research*, **11**, 81-99.

Bradshaw, D. J. & P. D. Marsh, 1998, Analysis of pH-driven disruption of oral microbial communities *in vitro*. *Caries Research*, **32**, 456-462.

Brown, D. F. J., 1994, Developments in antimicrobial susceptibility testing. *Reviews in Medical Microbiology*, **5**, 65-75.

Brown, N. L., Stoyanov, J. V., Kidd, S. P. & J. L. Hobman, 2003a, The MerR family of transcriptional regulators. *FEMS Microbiology Reviews*, **27**, 145-163.

Brown, N. L., Shih, Y. C., Leang, C., Glendinning, K. J., Hobman, J. L. & J. R. Wilson, 2003b, Mercury transport and resistance. *Biochemical Society Transactions*, **30**, 715-718.

Brozel, V. S., Pietersen, B. & T. E. Cloete, 1993, Adaptation of bacterial cultures to non-oxidising water treatment bactericide treatments. *Water SA (South Africa)*, **19**, 259-262.

Brumfitt, W. & J. M. Hamilton-Miller, 1988, The changing face of chemotherapy. *Postgraduate Medical Journal*, **64**, 552-558.

Brumfitt, W. & J. Hamilton-Miller, 1989, Methicillin-resistant *Staphylococcus aureus*. *New England Journal of Medicine*, **320**, 1188-1196.

Brune, D., 1981, Corrosion of amalgams. *Scandinavian Journal of Dental Research*, **89**, 506-514.

Bryskier, A., 2002, Viridans group streptococci: a reservoir of resistant bacteria in oral cavities. *Clinical Microbiology and Infection*, **8**, 65-69.

Burdett, V., 1986, Streptococcal tetracycline resistance mediated at the level of protein synthesis. *Journal of Bacteriology*, **165**, 564-569.

Burrows, D., 1986, Hypersensitivity to mercury, nickel and chromium in relation to dental materials. *International Dental Journal*, **36**, 30-34.

Burrus, V., Pavlovic, G., Decaris, B. & G. Guedon, 2002, Conjugative transposons: the tip of the iceberg. *Molecular Microbiology*, **46**, 601-610.

Carratalá, J., Alcaide, F., Fernández-Sevilla, A., Corbella, X., Liñares J. & F. Gudiol, 1995, Bacteremia due to viridans streptococci that are highly resistant to penicillin: increase among neutropenic patients with cancer. *Clinical Infectious Diseases*, **20**, 1169-1173.

Cerning, J., 1990, Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiology Reviews*, **7**, 113-130.

Chadwick, B. L., 1994, Products for prevention during orthodontics. *British Journal of Orthodontics*, **21**, 395-398.

Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J. & A. G. Sanders, 1940, Penicillin as a chemotherapeutic agent. *Lancet*, **ii**, 226-228. Also found on the Ameican Society for Microbiology website: http://www.asm.org/ASM/files/CCLIBRARYFILES/FILENAME/0000000263/21-3flemingchainabraham.pdf.

Chen, C., Ashimoto, A, Sangsurasak, S., Flynn, J. M. & J. Slots, 1997, Oral food consumption and subgingival microorganisms: subgingival microbiota of gastrostomy tube-feed children and healthy controls. *Journal of Periodontology*, **68**, 1163-1168.

Cherubin, C. E., Corrado, M. L., Sierra, M. F., Gombert, M. E. & M. Shulman, 1981, Susceptibility of Gram-positive cocci to various antibiotics. *Antimicrobial Agents and Chemotherapy*, **20**, 553-555.

Chopra, I. & M. Roberts, 2001, Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews*, **65**, 232-260.

Chung, W. O., Gabany, J., Persson, G. R. & M. C. Roberts, 2002, Distribution of *erm(F)* and *tet(Q)* genes in 4 oral bacterial species and genotypic variation between resistant and susceptible isolates. *Journal of Clinical Periodontology*, **29**, 152-158.

Church, D. L., Bryant, R. D., Sim, V. & E. J. Lishey, 1996, Metronidazole susceptibility and the presence of hydrogenase in pathogenic bacteria. *Anaerobe*, **2**, 147-153.

Clancy, J., Dib-Hajj, F., Petitpas, J. W. & W. Yuan, 1997, Cloning and characterization of a novel macrolide efflux gene, *mreA*, from *Streptococcus agalactiae*. *Antimicrobial Agents and Chemotherapy*, **41**, 2719-2723.

Clarkson, T. W., 1990, Mercury – an element of mystery. *New England Journal of Medicine*, **323**, 1137-1139.

Corbin, S. B. & W. G. Kohn, 1994, The benefits and risks of dental amalgam: current findings reviewed. *Journal of the American Dental Association*, **125**, 381-388.

Corpet, D. E., 1993, Antibiotic resistance from food. *New England Journal of Medicine*, **318**, 1206-1207.

Courvalin, P., 1994, Transfer of antibiotic resistance genes between Gram-positive and Gram-negative bacteria. *Antimicrobial Agents and Chemotherapy*, **38**, 1447-1451.

Courvalin, P., 1996, The Garrod lecture – evasion of antibiotic action by bacteria. *Journal of Antimicrobial Chemotherapy*, **37**, 855-869.

Craig, W. A., 1998, Pharmacokinetic/Pharmacodynamic parameters: Rationale for antibacterial dosing of mice and men. *Clinical Infectious Diseases*, **26**, 1-12.

Curtis, R. V. & D. Brown, 1992, The use of dental amalgam – an art or a science. *Dental Update*, **19**, 239-242, 244-245.

Cvitkovitch, D. G., 2001, Genetic competence and transformation in oral streptococci. *Critical Reviews in Oral Biology and Medicine*, **12**, 217-243.

Daito, M., Tanaka, T. & T. Hieda, 1992, Clinical observations on the development of third molars. *Journal of Osaka Dental University*, **26**, 91-104.

De Jong, M. H. & J. S. Van der Hoeven, 1987, The growth of oral bacteria on saliva. *Journal of Dental Research*, **66**, 498-505.

de Jonge, B. L. M., Chang, S., Gage, D. & A. Tomasz, 1992, Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. *Journal of Biological Chemistry*, **267**, 11248-11254.

Delnomdedieu, M. & J. W. Allis, 1993, Interaction of inorganic mercury salts with model and red cell membranes: importance of lipid binding sites. *Chemistry and Biology Interactions*, **88**, 71-87.

Demain, A. L., 1992, Microbial secondary metabolism: a new theoretical frontier for academia, a new opportunity for industry. *Ciba Foundation Symposia*, **171**, 3-23.

Demain, A. L. & R. P. Elander, 1999, The beta-lactam antibiotics: past, present, and future. *Antonie Van Leeuwenhoek*, **75**, 5-19.

de Neeling, A. J., van Leeuwen, W. J., Schouls, L. M., Schot, C. S., van Veen-Rutgers, A., Beunders, A. J., Buiting, A. G. M., Hol, C., Ligtvoet, E. E. J., Petit, P. L., Sabbe, L. J. M., van Griethuysen, A. J. A. & J. D A. van Embden, 1998, Resistance of staphylococci in the Netherlands: surveillance by an electronic network during 1989-1995. *Journal of Antimicrobial Chemotherapy*, **41**, 93-101.

Deyloff, J. L. & C. C. Sanders, 1980, Inhibition of *Streptococcus mutans* by human plaque flora. *Journal of Dental Research*, **59**, 1953-1959.

Diaz-Mejia, J. J., Carbajal-Saucedo, A. & C. F. Amabile-Cuevas, 2002, Antibiotic resistance in oral commensal streptococci from healthy Mexicans and Cubans: resistance prevalence does not mirror antibiotic usage. *FEMS Microbiology Letters*, **217**, 173-176.

Diekema, D. J., Beach, M. L., Pfaller, M. A., Jones, R. N. & the SENTRY Participants Group, 2001, Antimicrobial resistance in viridans group streptococci among patients with and without diagnosis of cancer in the USA, Canada and Latin America. *Clinical Microbiology and Infection*, 7, 152-157.

Distefano, M. D., Moore, M. J. & C. T. Walsh, 1990, Active site of mercuric reductase resides at the subunit interface and requires Cys-135 and Cys-140 from one subunit and Cys-558 and Cys-559 from the adjacent subunit: evidence from *in vivo* and *in vitro* heterodimer formation. *Biochemistry*, **29**, 2703-2713.

Doern, G. V., Ferraro, M. J., Brueggemann, A. B. & K. L. Ruoff, 1996, Emergence of high rates of antimicrobial resistance among viridans group streptococci in the United States. *Antimicrobial Agents and Chemotherapy*, **40**, 891-894.

Domagk, G., 1935, Ein beitrag zur chemotherapie der bakteriellen infektionen. *Deutsche Medizinische Wochenschrift*, **61**, 250-253. Translation on the ASM website: http://www.asm.org/ASM/files/CCLIBRARYFILES/FILENAME/0000000267/1935p195.pdf.

Dowd, F. J., 1999, Saliva and dental caries. *Dental Clinics in North America*, **43**, 579-597.

Dowson, G. G., Coffrey, T. J., Kell, C. & R. A. Whiley, 1993, Evolution of penicillin resistance in *S. pneumoniae*. The role of *S. mitis* in the formation of low affinity PBP2B in *S. pneumoniae*. *Molecular Microbiology*, **9**, 635-643.

Drucker D. B. & M. Jolly, 1971, Sensitivity of oral micro-organisms to antibiotics. *British Dental Journal*, **131**, 442-424.

Dyke, K. G. & M. H. Richmond, 1967, Occurrence of various types of penicillinase plasmids among 'hospital' staphylococci. *Journal of Clinical Pathology*, **20**, 75-79.

Dyke, K. G. H., Parker, M. T. & M. H. Richmond, 1970, Penicillinase production and metal-ion resistance in *Staphylococcus aureus* cultures isolated from hospital patients. *Journal of Medical Microbiology*, **3**, 125-136.

Eady, E. A., 1998, Bacterial resistance in acne. *Dermatology*, **196**, 59-66.

Eady, E. A., Ross, J. I. & J. H. Cove, 1990, Multiple mechanisms of erythromycin resistance. *Journal of Antimicrobial Chemotherapy*, **26**, 461-465.

Ebisu, S., Kato, K., Kotani, S. & A. Misaki, 1975, Structural differences in fructans elaborated by *Streptococcus mutans* and *Streptococcus salivarius*. *Journal of Biochemistry*, **78**, 879-887.

Ebrahim, G. J., 1995, Bacterial resistance to antibiotics – Editorial. *Journal of Tropical Pediatrics*, **41**, 130-131.

Edlund, C., Björkman, L., Ekstrand, J., Sandborgh-Englund, G. & C. E. Nord, 1996, Resistance of the normal human microflora to mercury and antimicrobials after exposure to mercury from dental amalgam fillings. *Clinical Infectious Diseases*, **22**, 944-950.

Edwards, D. I., 1993, Nitroimidazole drugs – action and resistance mechanisms. I. Mechanisms of action. *Journal of Antimicrobial Chemotherapy*, **31**, 9-20.

Endtz, H. P, Mouton, J. W., DEN Hollander, J. G., VAN DEN Braak, N. & H. A. Verbrugh, 1997, Comparative *in vitro* activities of Trovafloxacin (CP-99,219) against 445 Gram-positive isolates from patients with endocarditis and those with other blood stream infections. *Antimicrobial Agents and Chemotherapy*, **41**, 1146-1149.

Epstein, J. B., Chin, E. A., Jacobson, J. J., Rishiraj, B. & N. Le, 1998, The relationships among fluoride, cariogenic oral flora, and salivary flow rate during radiation therapy. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology and Endodontology*, **86**, 286-292.

Ericsson H. M. & J. C. Sherris, 1971, Antibiotic sensitivity testing. Report of an international collaborative study. *Acta Pathologica et Microbiologica Scandinavica. Section B: Microbiology and Immunology*, **217**, 1-90.

Essa, A. M. M., Macaskie, L. E. & N. L. Brown, 2002, Mechanisms of mercury bioremediation. *Biochemical Society Transactions*, **30**, 672-674.

Essa, A. M., Julian, D. J., Kidd, S. P., Brown, N. L. & J. L. Hobman, 2003, Mercury resistance determinants related to Tn21, Tn1696, and Tn5053 in enterobacteria from the preantibiotic era. *Antimicrobial Agents and Chemotherapy*, **47**, 1115-1119.

Farrell, R. E., Germida, J. J. & P. M. Huang, 1990, Biotoxicity of mercury as influenced by mercury (II) speciation. *Applied and Environmental Microbiology*, **56**, 3006-3016.

Featherstone, J. D., 1999, Prevention and reversal of dental caries: role of low level fluoride. *Community Dental and Oral Epidemiology*, **27**, 31-40.

Feder Jr, H. M. & R. A. Garibaldi, 1984, The significance of nongonococcal, nonmeningococcal *Neisseria* isolates from blood cultures. *Reviews of Infectious Diseases*, **6**, 181-188.

Ferracane, J. L., 1995, Current trends in dental composites. *Critical Reviews in Oral and Biological Medicine*, **6**, 302-318.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J.,Tomb, J-F., Dougherty, B. A., Merrick, J. M., McKenny, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J. D., Scott, J., Shirley, R., Liu, L-I., Glodek, A., Kelley, J. M., Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghagen, N. S. M., Gnehm, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O. & J. C. Ventner, 1995, Whole genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science*, **269**, 496-512.

Fleming, A., 1929, On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. *British Journal of Pathology*, **10**, 226-236. Found on the American Society for Microbiology website: http://www.asm.org/ASM/files/CCLIBRARYFILES/FILENAME/0000000263/21-3flemingchainabraham.pdf.

Freeman, C. D., Klutman, N. E. & K. C. Lamp, 1997, Metronidazole – a therapeutic review and update. *Drugs*, **54**, 679-708.

French, G. L., Ling, J. L., Hui, Y. W. & H. K. Oo, 1987, Determination of methicillin-resistance in *Staphylococcus aureus* by agar dilution and disc diffusion methods. *Journal of Antimicrobial Chemotherapy*, **20**, 599-608.

Gaffar, A., Afflitto, J. & N. Nabi, 1997, Chemical agents for the control of plaque and plaque microflora: an overview. *European Journal of Oral Science*, **105**, 502-507.

Garrod L. P. & P. M. Waterworth, 1971, A study of antibiotic sensitivity testing with proposals for simple uniform methods. *Journal of Clinical Pathology*, **24**, 779-789.

Gaustad, P., 1985, Genetic transformation in *Streptococcus sanguis*. Identification, surface spreading and competence of invasive strains of *Streptococcus sanguis* Lancefield groups H and W and other invasive viridans streptococci. *Acta Pathologica et Microbiologica Scandinavica. Section B: Microbiology and Immunology*, **93**, 277-282.

Georgopapadakou, N. H., 1993, Penicillin-binding proteins and bacterial resistance to β-lactams. *Antimicrobial Agents and Chemotherapy*, **37**, 2045-2053.

Gershon, A. S., de Azavedo, J. C. S., McGeer, A., Ostrowska, K. I., Church, D., Hoban, D. J., Harding, G. K. M., Weiss, K., Abbott, L., Smaill, F., Gpirdeau, M., Murray, G. & E. E. Low, 2002, Activities of new fluoroquinolones, ketolides, and other antimicrobials against blood culture isolates of viridans group streptococci from across Canada, 2000. *Antimicrobial Agents and Chemotherapy*, **46**, 1553-1556.

Geurtsen, W., 1998, Substances released from dental resin composites and glass ionomer cements. *European Journal of Oral Science*, **106**, 687-695.

Gibbons, R. J., 1968, Formation and significance of bacterial polysaccharides in caries etiology. *Caries Research*, **2**, 164-171.

Gilthorpe, M. S. & S. J. Cunningham, 2000, The application of multilevel, multivariate modelling to orthodontic research data. *Community Dental Health*, **17**, 236-242.

Girault, L., Lemaire, P., Boudou, A., Debouzy, J. C. & E. J. Dufourc, 1996, Interactions of inorganic mercury with phospholipid micelles and model membranes. A 31P-NMR study. *European Biophysics Journal*, **24**, 413-421.

Gold, O. G., Jordan, H. V. & J. van Houte, 1973, A selective medium for *Streptococcus mutans*. *Archives of oral biology*, **18**, 1357–1364.

Goldstein, F. W. & J. Garau, 1994, Resistant pneumococci: a renewed threat in respiratory infections. *Scandinavian Journal of Infectious Diseases*, **93**, 55-62.

Gordon, K. A., Beach, M. L., Biedenbach, D. J., Jones, R. N., Rhomberg, P. R. & A. H. Mutnick, 2002, Antimicrobial susceptibility patterns of β-hemolytic and viridans group streptococci: report from the SENTRY Antimicrobial Surveillance Program (1997-2000). *Diagnostic Microbiology and Infectious Disease*, **43**, 157-162.

Greenhalgh, T., 1986, Drug marketing in the third world: beneath the cosmetic reforms. *Lancet*, **I**, 1318-1320.

Greenstein, G., 1993, The role of metronidazole in the treatment of periodontal diseases. *Journal of Periodontology*, **64**, 1-15.

Gristina, A. G., 1987, Biomaterial-centered infection: Microbial adhesion versus tissue integration. *Science*, **237**, 1588-1595.

Grohmann, E., Muth, G. & M. Espinosa, 2003, Conjugative plasmid transfer in gram-positive bacteria. *Microbiology and Molecular Biology Reviews*, **67**, 277-301.

Groves, D. J., Short, H., Thewaini, A. J. & F. E. Young, 1975, Epidemiology of antibiotic and heavy metal resistance in bacteria: resistance patterns in staphylococci isolated from populations in Iraq exposed and not exposed to heavy metals or antibiotics. *Antimicrobial Agents and Chemotherapy*, **7**, 622-628.

Guggenheim, B., Lutz, F. & R. Schmid, 1997, Caries and plaque inhibition in rats by five topically applied dentifrices. *European Journal of Oral Science*, **105**, 258-263.

Gupta, A., Maynes, M. & S. Silver, 1998, Effects of halides on plasmid-mediated silver resistance in *Escherichia coli*. *Applied and Environmental Microbiology*, **64**, 5042-5045.

Gutierrez, J. H., Jofre, A. & F. Villena, 1990, Scanning electron microscope study on the action of endodontic irrigants on bacteria invading the dental tubules. *Oral Surgery, Oral Medicine and Oral Pathology*, **69**, 491-501.

Haefeli, C., Franklin, C. & K. Hardy, 1984, Plasmid-determined silver resistance in *Pseudomonas stutzeri* isolated from a silver mine. *Journal of Bacteriology*, **158**, 389-392.

Hahn, L. J., Kloiber, R., Vimy, M. J., Takahashi, Y. & F. L. Lorscheider, 1989, Dental 'silver' tooth fillings: A source of mercury exposure revealed by whole-body image scan and tissue analysis. *FASEB Journal*, **3**, 2641-2646.

Hall, B. M., 1970a, Mercury resistance of *Staphylococcus aureus*. *Journal of Hygiene*, **68**, 121-129.

Hall, B. M., 1970b, Distribution of mercury resistance among *Staphylococcus aureus* isolated from a hospital community. *Journal of Hygiene*, **68**, 111-119.

Hall, R. M. & C. M. Collis, 1998, Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resistance Updates*, **1**, 109-119.

Halsey, N. A., 1999, Limiting infant exposure to thimerosal in vaccines and other sources of mercury. *JAMA*, **282**, 1763-1766.

Hamlett, N. V., Landale, E. C., Davis, B. H. & A. O. Summers, 1992, Role of the Tn21, *merT*, *merP* and *merC* gene products in mercury resistance and mercury binding. *Journal of Bacteriology*, **174**, 6377-6385.

Hannula, J., Saarela, M., Jousimies-Somer, H., Takala, A., Syrjänen, R., Könönen, E. & S. Asikainen, 1999, Age-related acquisition of oral and nasopharyngeal yeast species and stability of colonization in young children. *Oral Microbiology and Immunology*, **14**, 176-182.

Hardie, J. M., 1992, Oral microbiology: current concepts in the microbiology of dental caries and periodontal disease. *British Dental Journal*, **172**, 271-278.

Haren, L., Ton-Hoang, B. & M. Chandler, 1999, Integrating DNA: tranposases and retroviral integrases. *Annual Reviews in Microbiology*, **53**, 245-281.

Hart, M. C., Elliott, G. N., Osborn, M., Ritchie, D. A. & P. Strike, 1998, Diversity among *Bacillus merA* genes amplified from mercury resistant isolates and directly from mercury polluted soil. *FEMS Microbiology Ecology*, **27**, 73-84.

Hayes, M. L., 1994, Reduction in acid tolerance of *Streptococcus downeii* by fluoride and potassium laurate. *Caries Research*, **28**, 94-98.

Heiddel, S., Sverrisson, J. T., Yngvason, F. E., Cariglia, N. & K. G. Kristinsson, 1993, Native-valve endocarditis due to *Neisseria sicca*: case report and review. *Clinical Infectious Diseases*, **16**, 667-670.

Heintze, S. D., Jost-Brinkmann, P. G. & J. Loundos, 1996, Effectiveness of three different types of electric toothbrushes compared with a manual technique in orthodontic patients. *American Journal of Orthodontics and Dentofacial Orthopediatrics*, **110**, 630-638.

Henriette, C., Petitdemange, E., Raval, G. & R. Gay, 1991, Mercuric reductase activity in the adaptation to cationic mercury, phenyl mercuric acetate and multiple antibiotics of a gram-negative population isolated from an aerobic fixed-bed reactor. *Journal of Applied Bacteriology*, **71**, 439-444.

Henwood, C. J., Livermore, D. M., Johnson, A. P., James, D., Warner, M., Gardiner, A. & the Linezolid Study Group, 2000, Susceptibility of gram-positive cocci from 25 UK hospitals to antimicrobial agents including linezolid. *Journal of Antimicrobial Chemotherapy*, **46**, 931-940.

Herp, A., Borelli, C. & A. M. Wu, 1988, Biochemistry and lectin binding properties of mammalian salivary mucous glycoproteins. *Advances in Experimental Medical Biology*, **228**, 395-435.

Hiramatsu, K., Hanaki, H., Ino, T., Oguri, T. & F. Tenover, 1998, Methicillin resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *Journal of Antimicrobial Agents and Chemotherapy*, **40**, 135-136.

Hobman, J. L. & N. L. Brown, 1997, Bacterial mercury-resistance genes. *Metal Ions in Biological Systems*, **34**, 527-568.

Hobman J. L., Essa A. M. M. & N. L. Brown, 2003, Mercury resistance (*mer*) operons in enterobacteria. *Biochemical Society Transactions*, **30**, 719-722.

Holland, H. D., 1998, Geochemistry – evidence for life on Earth more than 3850 million years ago. *Science*, **275**, 38-39.

Hse, K. M., Leung, S. K. & S. H. Wei, 1999, Resin-ionomer restorative materials for children: a review. *Australian Dental Journal*, **44**, 1-11.

Huang, C. C., Narita, M., Yamagata, T., Itoh, Y. & G. Endo, 1999, Structure analysis of a class II transposon encoding the mercury resistance of Gram-positive bacterium *Bacillus megaterium* MB a strain isolated from Minamata Bay, Japan. *Gene*, **234**, 361-369.

Hughes, V. M. & N. Datta, 1983, Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature*, **302**, 725-726.

Hughes, C. V., Kolenbrander, P. E., Anderson, R. N. & L. V. Moore, 1988, Coaggregation properties of human oral *Veillonella* species: relationship to colonization site and oral ecology. *Applied and Environmental Microbiology*, **54**, 1957-1963.

Huycke, M. M., Sahm, D. F. & M. S. Gilmore, 1998, Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. *Emerging Infectious Diseases*, **4**, 239-249.

Ieven, M., Goossens, W., De Wit, S. & H. Goossens, 2000, *In vitro* activity of gemifloxacin compared with other antimicrobial agents against recent clinical isolates of streptococci. *Journal of Antimicrobial Chemotherapy*, **45** Supplement S1, 51-53.

Ingram, G. S., 1990, Chemical events during tooth dissolution. *Journal of Dental Research*, **69**, 634-636.

Ioannidou, S., Tassios, P. T., Kotsovili-Tseleni, A., Foustoukou, M., Legakis, N. J. & A. Vatopoulos, 2001, Antibiotic resistance rates and macrolide resistance phenotypes of viridans group streptococci from the oropharynx of healthy Greek children. *International Journal of Antimicrobial Agents*, **17**, 195-201.

Ismail, A. I., Tanzer, J. M. & J. L. Dingle, 1997, Current trends of sugar consumption in developing societies. *Community Dental and Oral Epidemiology* **25**, 438-443.

Jacobs, J. A. & E. E. Stobberingh, 1996, *In vitro* antimicrobial susceptibility of the *'Streptococcus milleri'* group (*Streptococcus anginosus, Streptococcus constellatus* and *Streptococcus intermedius*). *Journal of Antimicrobial Chemotherapy*, **37**, 371-375.

Jacobs, J. A., van Baar, G. J., London, N. H. H. J., Tjhie, J. H. T., Schouls, L. M. & E. E. Stobberingh, 2001, Prevalence of macrolide-resistance genes in clinical isolates of the *Streptococcus anginosus* (*'S. milleri'*) group. *Antimicrobial Agents and Chemotherapy*, **45**, 2375-2377.

Jacobs, M. R., 1999, Drug-resistant *Streptococcus pneumoniae*: rational antibiotic choices. *American Journal of Medicine*, **106**, 19S-25S.

Jacoby, G. A., 1994, Genetics of extended-spectrum beta-lactamases. *European Journal of Clinical Microbiology and Infectious Diseases*, **13** (Supplement 1), 2-11.

Jenkinson, H. F., 1994, Cell surface protein receptors in oral streptococci. *FEMS Microbiology Letters*, **121**, 133-140.

Jenkinson, H. F. & R. J. Lamont, 1997, Streptococcal adhesion and colonization. *Critical Reviews in Oral and Biological Medicine*, **8**, 175-200.

Johnson, A. P., Warner, M., Broughton, K., James, D., Efsratiou, A., George, R. C. & D. M. Livermore, 2001, Antibiotic susceptibility of streptococci and related genera causing endocarditis: analysis of UK reference laboratory referrals, January 1996 to March 2000. *British Medical Journal*, **322**, 395-396.

Johnson, N. W., 1993, Hygiene and health: the value of antiplaque agents in promoting oral health. *International Dental Journal*, **43** (4 Supplement 1), 375-386.

Jones, R. N. & M. A. Pfaller, 2000, Potencies of newer fluoroquinolones against viridans group streptococci isolated in 637 cases of bloodstream infection in the SENTRY antimicrobial surveillance program (1997 to 1999): beyond Canada! *Antimicrobial Agents and Chemotherapy*, **44**, 2922-2923.

Jordan, V. & P. H. Keyes, 1966, *In vitro* methods for the study of plaque formation and carious lesions. *Archives in Oral Biology*, **11**, 793-802.

Jorgensen, J. H., 1993, Selection criteria for an antimicrobial susceptibility testing system. *Journal of Clinical Microbiology*, **31**, 2841-2844.

Joyston-Bechal, S., Hayes, K., Davenport, E. S. & J. M. Hardie, 1992, Caries incidence, mutans streptococci and lactobacilli in irradiated patients during a 12-month preventative programme using chlorhexidine and fluoride. *Caries Research*, **26**, 384-390.

Kamma, J. J., Diamanti-Kipioti, A., Nakou, M. & F. J. Mitsis, 2000, Profile of subgingival microbiota in children with mixed dentition. *Oral Microbiology and Immunology*, **15**, 103-111.

Kaufhold, A. & E. Potgieter, 1993, Chromosomally mediated high-level gentamicin resistance in *Streptococcus mitis*. *Antimicrobial Agents and Chemotherapy*, **37**, 2740-2742.

Kawamura, Y., Hou, X. C., Sultanu, F., Miura, H. & T. Ezaki, 1995, Determination of the 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationship among members of the *Streptococcus*. *International Journal of Systematic Bacteriology*, **45**, 406-408.

Kawamura, Y., Whiley, R. A., Shu, S-E., Ezaki, E. & J. M. Hardie, 1999, Genetic approaches to the identification of the mitis group within the genus streptococcus. *Microbiology*, **145**, 2605-2613.

Kay, H. M. & M. Wilson, 1988, The *in vitro* effects of amine fluorides on plaque bacteria. *Journal of Periodontology*, **59**, 266-269.

Kelstrup, J., 1981, Extracellular polysaccharides of smooth and rough variants of *Streptococcus salivarius*. *Scandinavian Journal of Dental Research*, **89**, 374-383.

Kennedy, H. F., Gemmell, C. G., Bagg, J., Gibson, B. E. S. & J. R. Michie, 2001, Antimicrobial susceptibility of blood culture isolates of viridans streptococci: relationship to a change in empirical antibiotic therapy in febrile neutropenia. *Journal of Antimicrobial Chemotherapy*, **47**, 693-696.

Kholodii, G. Y., Yurieva, O. V., Lomovskaya, O. L., Gorlenko, Z. M., Mindlin, S. Z. & V. G. Nikiforov, 1993, Tn5053, a mercury resistance transposon with integron ends. *Journal of Molecular Biology*, **230**, 1103-1107.

Kholodii, G., Yurieva, O., Mindlin, S., Gorlenko, Z., Rybochkin, V. & V. Nikiforov, 2000, Tn5044, a novel Tn3 family transposon coding for temperature-sensitive mercury resistance. *Research in Microbiology*, **151**, 291-302.

Khor, S. Y. & M. Jegathesan, 1983, Heavy metal and disinfectant resistance in clinical isolates of Gram-negative rods. *South East Asian Journal of Tropical Medicine*, **14**, 199-203.

Kobayashi, I., Kanayama, A., Matsuzaki, K., Nishida, M., Nakatogawa, N. & A. Kaneko, 2003, High-level gentamicin-resistant isolates of oral streptococci and *Aerococcus* from blood specimens. *Journal of Infection and Chemotherapy*, **9**, 21-24.

Koh, J., Wilson, M., Vidic, J. & H. N Newman, 1986, Amoxycillin-resistant streptococci in dental plaque. *Microbios*, **45**, 41-53.

Kong, R., Mebazaa, A., Heitz, B., De Breil, D. A., Kiredjian, M., Raskine, L. & D. Payen, 1998, Case of triple endocarditis caused by *Rothia dentocariosa* and results of a survey in France. *Journal of Clinical Microbiology*, **36**, 309-310.

Kostyniak, P. J., 1998, Mercury as a potential hazard for the dental practitioner. *New York State Dental Journal*, **64**, 40-43.

Kotra, L. P. & S. Mobashery, 1998, β-lactam antibiotics, β-lactamases and bacterial resistance. *Bulletin of the Institute of Pasteur*, **96**, 139-150.

Krčméry, V., Spanik, S. & J. Trupl, 1996a, First report of vancomycin resistant *Streptococcus mitis* bacteremia in a acute leukemic patient after prophylaxis with quinolones and during treatment with vancomycin. *Journal of Chemotherapy*, **84**, 325-326.

Krčméry, V., Trupl, J., Drgona, L., Lacka, J., Kukuckova, E. & E. Oravcoca, 1996b, Nosocomial bacteremia due to vancomycin-resistant *Staphylococcus epidermidis* in four patients with cancer, neutropenia and previous treatment with vancomycin. *European Journal of Clinical Microbiology and Infectious Disease*, **10**, 233-238.

Kuramitsu, H. K. & L. Wondrack, 1983, Insoluble glucan synthesis by *Streptococcus mutans* serotype c strains. *Infection and Immunity*, **42**, 763-770.

Lacroix, J-M. & C. B. Walker, 1992, Streptomycin resistance gene and partial Tn3 transposon coding for a β-lactamase in periodontal strain of *Eikenella corrodens*. *Antimicrobial Agents and Chemotherapy*, **36**, 740-743.

Lacroix, J-M. & C. B. Walker, 1995, Detection and incidence of the tetracycline resistance determinant *tet(M)* in the microflora associated with adult periodontitis. *Journal of Periodontology*, **66**, 102-108.

Laddaga, R. A., Chu, L., Misra, T. K. & S. Silver, 1987, Nucleotide sequence and expression of the mercurial-resistance operon from *Staphylococcus aureus* plasmid pI258. *Proceedings of the National Academy of Science*, **84**, 5106-5110.

Lane, D.J, 1991, 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York, NY.

Latinwo, L. M., Donald, C., Ikediobi, C. & S. Silver, 1998, Effects of intracellular glutathione on sensitivity of *Escherichia coli* to mercury and arsenite. *Biochemical and Biophysical Research Communications*, **242**, 67-70.

Leclercq, R., Derlot, E., Weber, M. & P. Courvalin, 1989, Transferable vancomycin and teicoplanin resistance in *E. faecium*. *Antimicrobial Agents and Chemotherapy*, **33**, 10-15.

Leclercq, R. & P. Courvalin, 1991a, Intrinsic and unusual resistance to macrolide, lincosamide and streptogramin antibiotics in bacteria. *Antimicrobial Agents and Chemotherapy*, **35**, 1273-1276.

Leclercq, R. & P. Courvalin, 1991b, Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. *Antimicrobial Agents and Chemotherapy*, **35**, 1267-1272.

Leclercq, R., 2002, Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clinical Infectious Diseases*, **34**, 482-492.

Leinfelder, K. F., 1991, Dental amalgam alloys. *Current Opinion in Dentistry*, **1**, 214-217.

Leistevuo, J., Järvinen, H., Österblad, M., Leistevuo, T., Huovinen, P. & J. Tenovuo, 2000, Resistance to mercury and antimicrobial agents in *Streptococcus mutans* isolates from human subjects in relation to exposure to dental amalgam fillings. *Antimicrobial Agents and Chemotherapy*, **44**, 456-457.

Leistevuo, J., Leistevuo, T., Helenius, H., Pyy, L., Huovinen, P. & J. Tenovuo, 2002, Mercury in saliva and the risk exceeding limits for sewage in relation to exposure to amalgam fillings. *Archives of Environmental Health*, **57**, 366-370.

Leng, Z., Riley, D. E., Berger, R. E., Krieger, J. N. & M. C. Roberts, 1997, Distribution and mobility of the tetracycline resistance determinant *tetQ*. *Journal of Antimicrobial Chemotherapy*, **40**, 551-559.

Levy, S. B, 1997, Antibiotic resistance: an ecological imbalance. *Ciba Foundation Symposia*, **207**, 1-9.

Levy, S. B, 1998, Multidrug resistance – a sign of the times. *New England Journal of Medicine*, **19**, 1376-1378.

Li, Y. & P. W. Caulfield, 1995, The fidelity of initial acquisition of mutans streptococci by infants from their mothers. *Journal of Dental Research*, **74**, 681-685.

Lie, T., 1978, Ultrastructural study of early dental plaque formation. *Journal of Periodontal Research*, **13**, 391-409.

Liebert, A. A., Wireman, J., Smith, T. & A. O. Summers, 1997, The impact of mercury released from dental 'silver' fillings on antibiotic resistances in the primate oral and intestinal bacterial flora. *Metal Ions in Biological Systems*, **34**, 441-460.

Liebert, C. A., Hall, R. M. & A. O. Summers, 1999, Transposon Tn21, flagship of the floating genome. *Microbiology and Molecular Biology Reviews*, **63**, 507-522.

Liljemark, W. F. & C. Bloomquist, 1996, Human oral microbial ecology and dental caries and periodontal diseases. *Critical Reviews in Oral Biology and Medicine*, **7**, 180-198.

Listgarten, M. A., Mayo, H. E. & R. Tremblay, 1975, Development of dental plaque on epoxy resin crowns in man. A light and electron microscopic study. *Journal of Periodontology*, **46**, 10-26.

Livermore, D. M., 1995, β-lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews*, **8**, 557-584.

Livermore, D. M., 2003, Linezolid *in vitro*: mechanisms and antibacterial spectrum. *Journal of Antimicrobial Chemotherapy*, **51** (Supplement 2), ii9-ii16.

Lockhart, S. R., Joly, S., Vargas, K., Swails-Wenger, J., Enger, L. & D. R. Soll, 1999, Natural defenses against *Candida* colonization breakdown in the oral cavities of the elderly. *Journal of Dental Research*, **78**, 857-868.

Loesche, W. J., 1968, Importance of nutrition in gingival crevice microbial ecology. *Periodontics*, **6**, 245-249.

Lorscheider, F. L. & M. J. Vimy, 1991, Mercury exposure from interior latex paint. *New England Journal of Medicine*, **324**, 851-852.

Lorscheider, F. L., Vimy, M. J., Summers, A. O., & H. Zwiers, 1995a, The dental amalgam mercury controversy - inorganic mercury and the CNS; genetic linkage of mercury and antibiotic resistances in intestinal bacteria. *Toxicology*, **97**, 19-22.

Lorscheider, F. L., Vimy, M. J. & A. O. Summers, 1995b, Mercury exposure from 'silver' tooth fillings: Emerging evidence questions a traditional dental paradigm. *FASEB Journal*, **9**, 504-508.

Lucas, V. S., Beighton, D. & G. J. Roberts, 2000, Composition of the oral streptococcal flora in healthy children. *Journal of Dentistry*, **28**, 45-50.

Luh, K-T., Hsueh, P-R, Teng, L-J., Pan, H-J., Chen, Y-C., Lu, J-J., Wu, J-J. & S-W. Ho, 2000, Quinupristin-Dalfopristin resistance among Gram-positive bacteria in Taiwan. *Antimicrobial Agents and Chemotherapy*, **44**, 3374-3380.

Luna, V. A., Coates, P., Eady, E. K., Cove, J. H., Nguyen, T. T. H. & M. C. Roberts, 1999, A variety of Gram-positive bacteria carry mobile *mef* genes. *Journal of Antimicrobial Chemotherapy*, **44**, 19-25.

Lyttle, H. A. & G. H. Bowden, 1993a, The level of mercury in human dental plaque and interaction *in vitro* between biofilms of *Streptococcus mutans* and dental amalgam. *Journal of Dental Research*, **72**, 1320-1324.

Lyttle, H. A. & G. H. Bowden, 1993b, The resistance and adaptation of selected oral bacteria to mercury and its impact on their growth. *Journal of Dental Research*, **72**, 1325-1330.

MacGowen, A. P. & R. Wise, 2001, Establishing MIC breakpoints and the interpretation of *in vitro* susceptibility tests. *Journal of Antimicrobial Chemotherapy*, **48** (Supplement 1), 17-28.

Mahler, I., Levinson, H. S., Wang, Y. & H. O. Halvorson, 1986, Cadmium- and mercury-resistant *Bacillus* strains from a salt marsh and from Boston harbor. *Applied and Environmental Microbiology*, **52**, 1293-1298.

Manganelli, R., Romano, L., Ricci, S., Zazzi, M. & G. Pozzi, 1995, Dosage of *Tn916* circular intermediates in *Enterococcus faecalis*. *Plasmid*, **34**, 48-57.

Maplestone, R. A., Stone, M. J. & D. H. Williams, 1992, The evolutionary role of secondary metabolites – a review. *Gene*, **115**, 151-157.

Marahiel, M. A., Nakano, M. M. & P. Zuber, 1993, Regulation of peptide antibiotic production in *Bacillus*. *Molecular Microbiology*, **7**, 631-636.

Marquis, R. E., 1995, Antimicrobial actions of fluoride for oral bacteria. *Canadian Journal of Microbiology*, **41**, 955-964.

Marsh, P. D., 1989, Host defenses and microbial homeostasis: Role of microbial interactions. *Journal of Dental Research*, **68** (Special Issue), 1567-1575.

Marsh, P. D., 1993, Antimicrobial strategies in the prevention of dental caries. *Caries Research*, **27** (Supplement 1), 72-76.

Marsh, P.D. & D. J. Bradshaw, 1997, Physiological approaches to the control of oral biofilms. *Advances in Dental Research*, **11**, 176-185.

Maskell, J. P., Sefton, A. M., Cannell, H., Kerawala, C., Seymour, A., Sun, Z. M. & J. D. Williams, 1990, Predominance of resistant oral streptococci in saliva and the effect of a single course of josamycin or erythromycin. *Journal of Antimicrobial Chemotherapy*, **26**, 539-548.

Massova, I. & S. Mobashery, 1998, Kinship and diversification of bacterial penicillin-binding proteins and β-lactamases. *Antimicrobial Agents and Chemotherapy*, **42**, 1-17.

Mayrand, D., McBride, B. C., Edwards T. & S. Jensen, 1980, Characterization of *Bacteroides asaccharolyticus* and *Bacteroides melaninogenicus* oral isolates. *Canadian Journal of Microbiology*, **26**, 1178-1183.

McCarthy, C., Snyder, M. L. & R. B. Parker, 1965, The indigenous oral flora of man - I - The newborn to the 1-year-old infant. *Archives in Oral Biology*, **10**, 61-70.

McNee, S. G., Geddes, D. A. & D. A. Weetman, 1982, Diffusion of sugars and acids in human dental plaque *in vitro*. *Archives in Oral Biology*, **27**, 975-979.

Messenger, A. J. M. & J. M. Turner, 1981, Effect of secondary metabolite production on the growth rate and variability of a pseudomonad. *Society of General Microbiology Quarterly*, **8**, 263-264.

Mettraux, G. R., Gusberti, F. A. & H. Graf, 1984, Oxygen tension (pO_2) in untreated human periodontal pockets. *Journal of Periodontology*, **55**, 516-521.

Michelich, V. J., Schuster, G. S. & D. H. Pashley, 1980, Bacterial penetration of human dentin *in vitro*. *Journal of Dental Research*, **59**, 1398-1403.

Miller, R. V., 1998, Bacterial gene swapping in nature. *Scientific American,* **278**, 66-71.

Miller, Y. W., Eady, E. A., Lacey, R. W., Cove, J. H., Joanes, D. N. & W. J. Cunliffe, 1996, Sequential antibiotic therapy for acne promotes the carriage of resistant staphylococci on the skin of contacts. *Journal of Antimicrobial Chemotherapy*, **38**, 829-837.

Millsap, K. W., Bos, R., van der Mei, H. C. & H. J Busscher, 1999, Adhesion and surface-aggregation of *Candida albicans* from saliva on acrylic surfaces with adhering bacteria as studied in a parallel plate flow chamber. *Antonie Van Leeuwenhoek*, **75**, 351-359.

Misra, T. K., Brown, N. L., Fritzinger, D. C., Pridmore, R. D., Barnes, W. M., Haberstroh, L. & S. Silver, 1984, The mercuric-ion resistance operons of plasmid R100 and transposon Tn501: the beginning of the operon including the regulatory region and the first two structural genes. *Proceedings of the National Academy of Science*, **81**, 5975-5979.

Misra, T. K., 1992, Bacterial resistances to inorganic mercury salts and organomercurials. *Plasmid*, **27**, 4-16.

Miyagi, A., Ohta, H., Kodama, T., Fukui, K., Kato, K. & T. Shimono, 1994, Metabolic and energetic aspects of the growth response of *Streptococcus ratus* to environmental acidification in anaerobic continuous culture. *Microbiology*, **140**, 1945-1952.

Moberg, L. E., 1988, Corrosion of dental amalgam and mercury vapour emission *in vitro*. *Scandinavian Journal of Oral Science*, **106**, 687-695.

Mohan, A., Morse, D. E., O'Sullivan, D. M. & N. Tinanoff, 1998, The relationship between bottle usage/content, age, and number of teeth with mutans streptococci colonization in 6-24-month-old children. *Community Dental and Oral Epidemiology*, **26**, 12-20.

Moore, B., 1960, A new screen test and selective medium for the rapid detection of epidemic strains of *Staph. aureus*. *Lancet*, **ii**, 453-458.

Moore, W. E., Holdeman, L. V., Smibert, R. M., Hash, D. E., Burmeister, J. A., Ranney, R. R., Savitt, E. D. & S. S. Socransky, 1984, Distribution of certain subgingival microbial species in selected periodontal conditions. *Journal of Periodontal Research*, **19**, 111-123.

Morhart, R. E. & R. J. Fitzgerald, 1976, Nutritional determinants of the ecology of the oral flora. *Dental Clinics in North America*, **20**, 473-489.

Morris, A., Kellner, J. D. & D. E. Low, 1998, The superbugs: evolution, dissemination and fitness. *Current Opinion in Microbiology*, **1**, 524-529.

Mukhopadhyay, D., Yu, H. R., Nucifora, G. & T. K. Misra, 1991, Purification and functional characterisation of MerD. A coregulation of the mercury resistance operon in gram-negative bacteria. *Journal of Biological Chemistry*, **266**, 18538-18542.

Naiman, R. A. & J. G. Barrow, 1963, Penicillin-resistant bacteria in the mouths and throats of children receiving continuous prophylaxis against rheumatic fever. *Annals of Internal Medicine*, **58**, 768-772.

Nakahara, H., Ishikawa, T., Sarai, Y., Kondo, I. & S. Mituhashi, 1977a, Frequency of heavy-metal resistance in bacteria from inpatients in Japan. *Nature*, **266**, 165-167.

Nakahara, H., Ishikawa, T., Sarai, Y., Kondo, I., Kozukue, H. & S. Silver, 1977b, Linkage of mercury, cadmium and arsenate and drug resistance in clinical isolates of *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*, **33**, 975-976.

Nakano, M. M. & P. Zuber, 1990, Molecular biology of antibiotic production in *Bacillus*. *Critical Reviews in Biotechnology*, **10**, 223-240.

Narita, M., Chiba, K., Nishizawa, H., Ishii, H., Huang, C. C., Kawabata, Z., Silver, S.& G. Endo, 2003, Diversity of mercury resistance determinants among Bacillus strains isolated from sediment of Minamata Bay. *FEMS Microbiology Letters*, **223**, 73-82.

Nascimento, A. M., Campos, C. E., Campos, E. P., Azevedo, J. L. & E. Chartone-Souza, 1999, Re-evaluation of antibiotic and mercury resistance in *Escherichia coli* populations isolated in 1978 from Amazonian rubber tree tappers and Indians. *Research in Microbiology*, **150**, 407-411.

Nascimento, A. M. A. & E. Chartone-Souza, 2003, Operon *mer*: Bacterial resistance to mercury and potential for bioremediation of contaminated environments. *Genetics and Molecular Research*, **2**, 92-101.

Neu, H. C., 1992, The crisis in antibiotic resistance. *Science*, **257**, 1064-1073.

Newbrun, E., 1992, Dental caries in the future: a global view. *Proceedings of the Finnish Dental Society*, **88**, 155-161.

Nikiforov, V. G., Bass, I. A., Bogdanova, E. S., Gorlenko, Z. M., Kalyaeva, E. S., Kopteva, A. V., Lomovskaya, O. L., Minakhin, L. S., Minakhina, S. V., Mindlin, S. Z., Petrova, M. A., Kholodii, G. Y. & O. V. Yurieva, 1999, Distribution of mercury resistance transposons in environmental bacterial populations. *Molecular Biology*, **33**, 44-50.

Novick, R. P., 1963, Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. *Journal of General Microbiology*, **33**, 121-136.

Olsen, J. E., 1999, Antibiotic resistance: genetic mechanisms and mobility. *Acta Veterinaria Scandinavica*, Supplement **92**, 15-22.

Ono, T., Shiota, S., Hirota, K., Nemoto, K., Tsuchiya, T. & Y. Miyake, 2000, Susceptibilities of oral and nasal isolates of *Streptococcus mitis* and *Streptococcus oralis* to macrolides and PCR detection of resistance genes. *Antimicrobial Agents and Chemotherapy*, **44**, 1078-1080.

Oppenheim, B. A., 1998, The changing pattern of infection in neutropenic patients. *Journal of Antimicrobial Chemotherapy*, **41** Supplement D, 7-11.

Osborn, A. M., Bruce, K. D., Strike, P. & D. A. Ritchie, 1997, Distribution, diversity and evolution of the bacterial mercury resistance (*mer*) operon. *FEMS Microbiology Reviews*, **19**, 239-262.

Österblad, M., Leistevuo, J., Leistevuo, T., Järvinen, H., Pyy, L., Tenovuo, J. & P. Huovinen, 1995, Antimicrobial and mercury resistance in aerobic gram-negative bacilli in fecal flora among persons with and without dental amalgam fillings. *Antimicrobial Agents and Chemotherapy*, **39**, 2499-2502.

Packer, S., Woodley, N., Wilson, M. & P. Mullany, 1999, Prevalence and persistence of amoxycillin-resistant bacteria in the dental plaques of adults. *Microbios*, **100**, 135-144.

Pan-Hou, H. S. K. & N. Imura, 1981, Role of hydrogen sulfide in mercury resistance determined by plasmid of *Clostridium cochlearium* T-2. *Archives in Microbiology*, **129**, 49-52.

Papapetropoulou, M., Iliopoulou, J., Rodopoulou, G., Detorakis, J. & O. Paniara, 1994, Occurrence and antibiotic-resistance of *Pseudomonas* species isolated from drinking water in southern Greece. *Journal of Chemotherapy*, **6**, 111-116.

Pearce, C., Bowden, G. H., Evans, M., Fitzsimmons, S. P., Johnson, J., Sheridan, M. J., Wientzen, R. & M. F. Cole, 1995, Identification of pioneer viridans streptococci in the oral cavity of human neonates. *Journal of Medical Microbiology*, **42**, 67-72.

Petsko, G. A., 1991, Déjà vu all over again. *Nature*, **352**, 104-105.

Pfaller, M. A. & R. N. Jones, 1997, *In vitro* evaluation of contemporary β-lactam drugs tested against viridans groups and β-hemolytic streptococci. *Diagnostic Microbiology and Infectious Disease*, **27**, 151-154.

Philippon, A., Labia, R. & G. Jacoby, 1989, Extended-spectrum β-lactamases. *Antimicrobial Agents and Chemotherapy*, **33**, 1131-1136.

Piddock, L. J. V., 1998, Antibacterials – mechanisms of action. *Current Opinion in Microbiology*, **1**, 502-508.

Pike, R., Stapleton, P., Lucas, V., Roberts, G. J., Rowbury, R., Richards, H., Mullany, P. & M. Wilson, 2002a, Effect of medium composition on the susceptibility of oral streptococci to mercuric chloride. *Current Microbiology,* **45**, 272-276.

Pike, R., Lucas, V., Stapleton, P., Gilthorpe, M. S., Roberts, G., Rowbury, R., Richards, H., Mullany, P. & M. Wilson, 2002b, Prevalence and antibiotic resistance profile of mercury-resistant oral bacteria from children with and without mercury amalgam fillings. *Journal of Antimicrobial Chemotherapy*, **49**, 777-783.

Pike, R., Lucas, V., Petrie, A., Roberts, G., Stapleton, P., Rowbury, R., Richards, H., Mullany, P. & M. Wilson, 2003, Effect of restoration of children's teeth with mercury amalgam on the prevalence of mercury- and antibiotic-resistant oral bacteria. *Microbial Drug Resistance*, **9**, 93-97.

Porter, F. D., Silver, S., Ong, C. & H. Nakahara, 1982, Selection for mercurial resistance in hospital settings. *Antimicrobial Agents and Chemotherapy*, **22**, 852-858.

Post D. E. & E. Y. Bridson, 1991, Problems with Iso-Sensitest agar. *Journal of Antimicrobial Chemotherapy*, **27**, 867-868.

Potgieter, E., Carmichael, M., Koornhof, H. J. & L. J. Chalkley, 1992, *In vitro* antimicrobial susceptibility of viridans streptococci isolated from blood cultures. *European Journal of Clinical Microbiology and Infectious Disease*, **11**, 543-545.

Poutanen, S. M., DE Azavedo, J., Willey, B. M., Low, D. E. & K. S. MacDonald, 1999, Molecular characterization of multidrug resistance in *Streptococcus mitis*. *Antimicrobial Agents and Chemotherapy*, **43**, 1505-1507.

Quirynen, M., 1994, The clinical meaning of the surface roughness and the surface free energy of intra-oral hard substrata on the microbiology of the supra- and subgingival plaque: results on *in vitro* and *in vivo* experiments. *Journal of Dentistry*, **22** (Supplement 1), S13-S16.

Ranly, D. M., 1998, Early orofacial development. *Journal of Clinical Pediatric Dentistry*, **22**, 267-275.

Ready, D., Qureshi, F., Bedi, R., Mullany, P. & M. Wilson, 2003, Oral bacteria resistant to mercury and to antibiotics are present in children with no previous exposure to amalgam restorative materials. *FEMS Microbiology Letters*, **223**, 107-111.

Reller, L. B., Schoenknecht, F. D., Kenny, M. A. & J. C. Sherris, 1974, Antibiotic susceptibility testing of *Pseudomonas aeruginosa*: selection of a control strain and criteria for magnesium and calcium content in media. *The Journal of Infectious Diseases*, **130**, 454-463.

Renneberg J., Niemann, L. L. & E. Gutschik, 1997, Antimicrobial susceptibility of 278 streptococcal blood isolates to seven antimicrobial agents. *Journal of Antimicrobial Chemotherapy*, **39**, 135-140.

Reynolds, P. E., 1989, Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *European Journal of Clinical Microbiology & Infectious Diseases*, **8**, 943-950.

Richmond, M. H. & M. John, 1964, Co-transduction by a staphylococcal phage of the genes responsible for penicillinase synthesis and resistance to mercury salts. *Nature*, **202**, 1360-1361.

Riley, T. & B. Mee, 1982, Susceptibility of *Bacteroides* spp to heavy metals. *Antimicrobial Agents and Chemotherapy*, **22**, 889-892.

Riley T. V. & M. L. Taylor, 1989, A note on susceptibility of *Branhamella catarrhalis* to heavy metals. *Journal of Applied Bacteriology*, **67**, 185-189.

Roberts, M. C., 1994, Epidemiology of tetracycline-resistance determinants. *Trends in Microbiology*, **2**, 353-357.

Roberts, M. C., 1996, Tetracycline resistance determinants: Mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiology Review*, **19**, 1-24.

Roberts, M. C., 1998, Antibiotic resistance mechanisms in bacteria of oral and upper respiratory origin. *International Journal of Antimicrobial Agents and Chemotherapy*, **9**, 255-267.

Roberts, M. C., Sutcliffe, J., Courvalin, P., Jensen, L. B., Rood, J. & H. Seppala, 1999, Nomenclature for macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrobial Agents and Chemotherapy*, **43**, 2823-2830.

Rodriguez-Avial, I., Rodriguez-Avial, C., Culebras, E. & J. J. Picazo, 2003, Distribution of tetracycline resistance genes *tet*(M), *tet*(O), *tet*(L) and *tet*(K) in blood isolates of viridans group streptococci harbouring *erm*(B) and *mef*(A) genes. Susceptibility to quinupristin/dalfopristin and linezolid. *International Journal of Antimicrobial Agents*, **21**, 536-541.

Ronda, C., Garcia, J. L. & R. Lopez, 1988, Characterization of genetic transformation in *Streptococcus oralis* NCTC 11427: expression of the pneumococcal amidase in *S. oralis* using a new shuttle vector. *Molecular & General Genetics*, **215**, 53-57.

Rowe-Magnus, D. A. & D. Mazel, 2001, Integrons: natural tools for bacterial gene evolution. *Current Opinion in Microbiology*, **4**, 565-569.

Rowe-Magnus, D. A., Davies, J. & D. Mazel, 2002, Impact of integrons and transposons on the evolution of resistance and virulence. *Current Topics in Microbiology and Immunology*, **264**, 167-188.

Ruby, J. D., Goldner, M. & J. A. Hargraves, 1978, *Streptococcus mutans*, an assessment of its physiological potential in relation to dental caries. *Reviews in Canadian Biology*, **37**, 273-289.

Rudrik, J. T., Bawdon, R. E. & S. P. Guss, 1985, Determination of mercury and organomercurial resistance in obligate anaerobic bacteria. *Canadian Journal of Microbiology*, **31**, 276-281.

Russell, A. D., Tattawasart, U., Maillard, J-Y. & J. R. Furr, 1998, Possible link between resistance and use of antibiotics and biocides. *Antimicrobial Agents and Chemotherapy*, **42**, 2151.

Rykke, M., 1992, Dental materials for posterior restorations. *Endodontal Dentistry and Traumatology*, **8**, 139-148.

Sadhukhan, P. C., Ghosh, S., Chaudhuri, J., Ghosh, D. K. & A, Mandal, 1997, Mercury and organomercurial resistance in bacteria isolated from freshwater fish of wetland fisheries around Calcutta. *Environmental Pollution*, **97**, 71-78.

Salamon, S. A. & J. Prag, 2001, Three cases of *Rothia dentocariosa* bacteraemia: frequency in Demark and a review. *Scandinavian Journal of Infectious Diseases*, **34**, 153-157.

Salyers, A. A., Shoemaker, N. B., Stevens, A. M. & L-Y. Li, 1995, Conjugative transposons: an unusual and diverse set of integrated gene elements. *Microbiology Reviews*, **59**, 579-590.

Salyers, A. A. & N. B. Shoemaker, 1996, Resistance gene transfer in anaerobes: New insights, new problems. *Clinical Infectious Diseases*, **23** (Supplement 1), S36-S43.

Samuelson, J., 1999, Why is metronidazole active against both bacteria and parasites. *Antimicrobial Agents and Chemotherapy*, **43**, 1533-1541.

Schaeken, M. J., van der Hoeven, J. S. & H. C. Franken, 1986, Comparative recovery of *Streptococcus mutans* on five isolation media, including a new simple selective medium. *Journal of Dental Research*, **65**, 906-908.

Schatz, A., Bugie, E. & S. A. Waksman, 1944, Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. *Proceedings of the Society for Experimental Biology & Medicine,* **55**, 66-69.

Schiering, N., Kabsch, W., Moore, M. J., Distefano, M. D., Walsh, C. T. & E. F. Pai, 1991, Structure of the detoxification catalyst mercuric ion reductase from *Bacillus* sp. strain RC607. *Nature*, **352**, 168-172.

Schmitz, F-J., Fluit, A. C., Gondolf, M., Beyrau, R., Lindenlauf, E., Verhoef, J., Heinz, H-P. & M. E. Jones, 1999, The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. *Journal of Antimicrobial Chemotherapy*, **43**, 253-259.

Schottel, J., Mandal, A., Clark, D., Silver, S. & R. W. Hedges, 1974, Volatisation of mercury and organomercurials determined by inducible R-factor systems in enteric bacteria. *Nature*, **251**, 335-337.

Schuster, G. S., 1999, Oral flora and pathogenic organisms. *Infectious Disease Clinics in North America*, **13**, 757-774.

Schwalbe, R. S., Stapleton, J. T. & P.H. Gilligan, 1987, Emergence of vancomycin-resistance in coagulase-negative staphylococci. *New England Journal of Medicine*, **316**, 927-931.

Sedlmeier, R. & J. Altenbuchner, 1992, Cloning and DNA sequence analysis of the mercury resistance genes of *Streptomyces lividans*. *Molecular and General Genetics*, **236**, 76-85.

Sefton, A. M., 1999, Macrolides and changes in oral flora. *International Journal of Antimicrobial Agents*, **11** (Supplement 1), S23-S29; discussion S31-S32.

Seppala, H., Haanpera, M., Al-Juhaish, M., Jarvinen, H., Jalava, J. & P. Huovinen, 2003, Antimicrobial susceptibility patterns and macrolide resistance genes of viridans group streptococci from normal flora. *Journal of Antimicrobial Chemotherapy*, **52**, 636-644.

Shah, H. N. & M. D. Collins, 1983, Genus *Bacteroides*. A chemotaxonomical perspective. *Journal of Applied Bacteriology*, **55**, 403-416.

Shapiro, S., 1996, The inhibitory action of fatty acids on oral bacteria. *Oral Microbiology and Immunology*, **11**, 350-355.

Siblerud, R. L., Motl, J. & E. Kienholz, 1994, Psychometric evidence that mercury from silver dental fillings may be an etiological factor in depression, excessive anger, and anxiety. *Psychology Reports*, **74**, 67-80.

Silver, S., 1996, Bacterial resistances to toxic metal ions – a review. *Gene*, **179**, 9-19.

Silver, S., 2003, Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. *FEM Microbiology Reviews*, **27**, 341-353.

Silver S. & T. K. Misra, 1988, Plasmid-mediated heavy metal resistances. *Annual Review of Microbiology*, **42**, 717-743.

Silver, S. & M. Walderhaug, 1992, Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. *Microbiological Reviews*, **56**, 195-228.

Silver, S. & G. Ji, 1994, Newer systems for bacterial resistances to toxic heavy metals. *Environmental Health Perspectives*, **102**, 107-113.

Silver, S. & L. T. Phung, 1996, Bacterial heavy metal resistance: new surprises. *Annual Reviews in Microbiology*, **50**, 753-789.

Sirot, D., 1995, Extended-spectrum plasmid-mediated β-lactamases. *Journal of Antimicrobial Chemotherapy*, **36** (Supplement A), 19-34.

Slots, J., 1979, Subgingival microflora and periodontal disease. *Journal of Periodontology*, **6**, 351-382.

Smith, D. J., Anderson, J. M., King, W. F., van Houte, J. & M. A. Taubman, 1993, Oral streptococci colonization of infants. *Oral Microbiology and Immunology*, **8**, 1-4.

Smith, C. J., Tribble, G. D. & D. P. Bayley, 1998, Genetic elements of *Bacteroides* species: a moving story. *Plasmid*, **40**, 12-29.

Socransky, S. S., Gibbons, R. J., Dale, A. C., Bortnick, L., Rosenthal, E. & J. B. Mcdonald, 1963, The microbiota of the gingival crevice in man. *Archives in Oral Biology*, **8**, 275.

Socransky, S. S. & S. D. Manganiello, 1971, The oral microbiota of man from birth to senility. *Journal of Periodontology*, **42**, 485-494.

Socransky, S. S., Manganiello, A. D., Propas, D., Oram, V. & J. van Houte, 1977, Bacteriological studies of developing dental plaque. *Journal of Periodontal Research*, **12**, 107-119.

Speer, B. S., Bedzyk, L. & A. A. Salyers, 1991, Evidence that a novel tetracycline resistance gene found in two *Bacteroides* transposons encodes an NADP-requiring oxidoreductase. *Journal of Bacteriology*, **173**, 176-183.

Speer, B. S., Shoemaker, N. B. & A. A. Salyers, 1992, Resistance to tetracycline: Mechanisms and transmission. *Clinical Microbiology Reviews*, **5**, 387-399.

Spratt, B. G., 1988, Hybrid penicillin-binding proteins in penicillin-resistant isolates of *Neisseria gonorrhoeae*. *Nature*, **332**, 173-176.

Spratt, B. G., Bowler, L. D., Zhang, Q-Y., Zhou, J. & J. M. Smith, 1992, Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. *Journal of Molecular Evolution*, **34**, 115-125.

Srinivasan, A., Dick, J. D. & T. M. Perl, 2002, Vancomycin resistance in staphylococci. *Clinical Microbiology Reviews*, **15**, 430-438.

Stapleton, P., Adams, V., Pike., R., Lucas, V., Roberts, G., Mullany, P., Rowbury, R., Wilson, M. & H. Richards, 2004, Characterisation of viridans group streptococci with different levels of Tet(M)-mediated tetracycline resistance. *International Journal of Antimicrobial Agents*, **24**, 439-443.

Steers, E., Foltz, E. L. & B. S. Graves, 1959, An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. *Antimicrobial Agents and Chemotherapy*, **9**, 307-311.

Stone, M. J. & D. H. Williams, 1992, On the evolution of functional secondary metabolites (natural products). *Molecular Microbiology*, **6**, 29-34.

Stratton, C. W., 2002, Chloramphenicol. *Antimicrobics and Infectious Diseases Newsletter*, **18**, 89-91.

Summers, A. O., 1986, Organization, expression, and evolution of genes for mercury resistance. *Annual Reviews in Microbiology*, **40**, 607-634.

Summers A. O. & L. L. Sugarman, 1974, Cell-free mercury(II)-reducing activity in a plasmid-bearing strain of *Escherichia coli*. *Journal of Bacteriology*, **119**, 242-249.

Summers, A. O., Wireman, J., Vimy, M. J., Lorscheider, F. L., Marschall, B., Levy, S. B., Bennett, S. & L. Billard, 1993, Mercury released from dental 'silver' fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal flora of primates. *Antimicrobial Agents and Chemotherapy*, **37**, 825-834.

Sundqvist, G., 1994, Taxonomy, ecology, and pathogenicity of the root canal flora. *Oral Surgery, Oral Medicine and Oral Pathology*, **78**, 522-530.

Sutter, V. L., Jones, M. J. & A. T. M. Ghoneim, 1983, Antimicrobial susceptibilities of bacteria associated with periodontal disease. *Antimicrobial Agents and Chemotherapy*, **23**, 483-486.

Szabo, S., Lieberman, J. P. & Y. A. Lue, 1990, Unusual pathogens in narcotic-associated endocarditis. *Reviews of Infectious Diseases*, **12**, 412-415.

Tanaka, M., Hanioka, T., Takaya, K. & S. Shizukuishi, 1998, Association of oxygen tension in human periodontal pockets with gingival inflammation. *Journal of Periodontology*, **69**, 1127-1130.

Tattawasart, U., Maillard, J. Y., Furr, J. R. & A. D. Russell, 1999, Development of resistance to chlorhexidine diacetate and cetylpyridinium chloride in *Pseudomonas stutzeri* and changes in antibiotic susceptibility. *Journal of Hospital Infection*, **42**, 219-229.

Taylor, D. E. & A. Chau, 1996, Tetracycline resistance mediated by ribosomal protection. *Antimicrobial Agents and Chemotherapy*, **40**, 1-5.

Teng, L. J., Hsueh, R. R., Chen, Y. C., Ho, S. W. & K. T. Luh, 1998, Antimicrobial susceptibility of viridans group streptococci in Taiwan with an emphasis on the high rates of resistance to penicillin and macrolides in *Streptococcus oralis*. *Journal of Antimicrobial Chemotherapy*, **41**, 621-627.

Timoney, J. F., Port, J., Giles, J. & J. Spanier, 1978, Heavy-metal and antibiotic resistance in the bacterial flora of sediments of New York Bight. *Applied & Environmental Microbiology*, **36**, 465-472.

Tompkins, G. R., Peavey, M. A., Birchmeier, K. R. & J. R. Tagg, 1997, Bacteriocin production and sensitivity among coaggregating and noncoaggregating oral streptococci. *Oral Microbiolgy and Immunology*, **12**, 98-105.

Traub, W. H. & B. Leonhard, 1997, Antibiotic susceptibility of α- and nonhemolytic streptococci from patients and healthy adults to 24 antimicrobial drugs. *Chemotherapy*, **43**, 123-131.

Tunkel, A. R. & K. A. Sepkowitz, 2002, Infections caused by viridans streptococci in patients with neutropenia. *Clinical Infectious Diseases*, **34**, 1524-1529.

Tuohy, M. & J. A. Washington, 1997, Antimicrobial susceptibility of viridans group streptococci. *Diagnostic Microbiology and Infectious Disease*, **29**, 277-280.

Tyas, M. J., 1995, The class V lesion – aetiology and restoration. *Australian Dental Journal*, **40**, 167-170.

Uttley, A. H. C., Collins, C. H., Naidoo, J. & R. C. George, 1988, Vancomycin resistant enterococci. *Lancet*, **331**, 136.

van Houte, J., 1994, Role of micro-organisms in caries etiology. *Journal of Dental Research*, **73**, 672-681.

Van Winkelhoff, A. J., Gonzales, D. H., Winkel, E. G., Dellemijn-Kippuw, N., Vanden-broucke-Grauls, C. M. J. E. & M. Sanz, 2000, Antimicrobial resistance in the subgingival microflora in patients with adult periodontitis. *Journal of Clinical Periodontology*, **27**, 79-86.

Venditti, M., Baiocchi, P., Santini, C., Brandimarte, C., Serra, P., Gentile, G., Girmenia, C. & P. Martino, 1989, Antimicrobial susceptibilities of *Streptococcus* species that cause septicemia in neutropenic patients. *Antimicrobial Agents and Chemotherapy*, **33**, 580-585.

Vernazza, T. R. & T. H. Melville, 1979, Inhibitory activity of *Streptococcus mitis* against oral bacteria. *Microbios*, **26**, 95-101.

Villedieu, A., Diaz-Torres, M. L., Hunt, N., McNab, R., Spratt, D. A., Wilson, M. & P. Mullany, 2003, Prevalence of tetracycline resistance genes in oral bacteria. *Antimicrobial Agents and Chemotherapy*, **47**, 878-882.

Vimy, M. J. & F. L. Lorscheider, 1985a, Intra-oral air mercury released from dental amalgam. *Journal of Dental Research*, **64**, 1069-1071.

Vimy, M. J. & F. L. Lorscheider, 1985b, Serial measurements of intra-oral air mercury: Estimation of daily dose from dental amalgam. *Journal of Dental Research*, **64**, 1072-1075.

Wallace, B. L., Bradley, J. E. & M. Rogolsky, 1981, Plasmid analyses in clinical isolates of *Bacteroides fragilis* and other *Bacteroides* species. *Journal of Clinical Microbiology*, **14**, 383-388.

Wang, Y., Moore, M., Levinson, H. S., Silver, S., Wash, C. & I. Mahler, 1989, Nucleotide sequence of a chromosomal mercury resistance determinant from a *Bacillus* sp. with broad-spectrum mercury resistance. *Journal of Bacteriology*, **171**, 89-92.

Wasfy, M. O., Bajuscak, R. E., Santos, A. C. & G. E. Minah, 1986, β-lactamase resistance of black-pigmented bacteroides in gingival plaques of Egyptian children. *Journal of Periodontal Research*, **21**, 450-454.

Watanakunakorn, C. & J. Pantelakis, 1993, Alpha-hemolytic streptococcal bacteremia: a review of 203 episodes during 1980-1991. *Scandinavian Journal of Infectious Disease*, **25**, 403-408.

Ween, O., Teigen, S., Gaustad, P., Kilian, M & L. S. Havarstein, 2002, Competence without a competence pheromone in a natural isolate of *Streptococcus infantis*. *Journal of Bacteriology*, **184**, 3426-3432.

Weisblum, B., 1995, Erythromycin resistance by ribosome modification. *Antimicrobial Agents and Chemotherapy*, **39**, 577-585.

Westling, K. Ljungman, P., Thalme, A. & I. Julander, 2002, Streptococcus viridans septicaemia: a comparison study in patients admitted to the departments of infectious diseases and haematology in a university hospital. *Scandinavian Journal of Infectious Diseases*, **34**, 316-319.

Whatmore, A. M., Efstratiou, A., Pickerill, A. P., Broughton, K., Woodard, G., Sturgeon, D., George, R. & C. G. Dowson, 2000, Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*: characterization of "atypical" pneumococci and organisms allied to *S. mitis* harboring *S. pneumoniae* virulence factor-encoding genes. *Infection and Immunity*, **68**, 1374-1382.

Wheat, P. F., 2001, History and development of antimicrobial susceptibility testing methodology. *Journal of Antimicrobial Chemotherapy*, **47** (Supplement S1), 1-4.

Williams, R. J. & D. L. Heymann, 1998, Containment of antibiotic resistance. *Science*, **279**, 1153-1154.

Wilson, M., 1996, Susceptibility of oral bacterial biofilms to antimicrobial agents. *Journal of Medical Microbiology*, **44**, 79-87.

Wilson, J., 1998, The aetiology, diagnosis and management of denture stomatitis. *British Dental Journal*, **185**, 380-384.

Winder, A. F., Astbury, N. J., Sheraidah, G. A. & M. Ruben, 1980, Penetration of mercury from ophthalmic preservatives into the human eye. *Lancet*, **2**, 237-239.

Winston, A. E. & S. N. Bhaskar, 1998, Caries prevention in the 21st Century. *Journal of the American Dental Association*, **129**, 1579-1587.

Wireman, J., Liebert, C. A., Smith T. & A. O. Summers, 1997, Association of mercury resistance with antibiotic resistance in the Gram-negative fecal bacteria of primates. *Applied and Environmental Microbiology*, **63**, 4494-4503.

Wisplinghoff, H., Reinert, R. R., Cornely, O. & H. Seifert, 1999, Molecular relationships and antimicrobial susceptibilities of viridans group streptococci isolated from blood of neutropenic cancer patients. *Journal of Clinical Microbiology*, **37**, 1876-1880.

Witte, W., Green, L., Misra, T. K. & S. Silver, 1986, Resistance to mercury and cadmium in chromosomally resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, **29**, 663-669.

Woodford, N., 1998, Glycopeptide-resistant enterococci: a decade of experience. *Journal of Medical Microbiology*, **47**, 849-862.

Woodford, N., Johnson, A. P., Morrision, D. & D. C. E. Speller, 1995, Current perspectives on glycopeptide resistance. *Clinical Microbiology Reviews*, **8**, 585-615.

Woodford, N., 2001, Epidemiology of the genetic elements responsible for acquired glycopeptide resistance in enterococci. *Microbial Drug Resistance*, **7**, 229-36.

Woodward, M. & A. R. Walker, 1994, Sugar consumption and dental caries: evidence from 90 countries. *British Dental Journal*, **176**, 297-302.

Yan, J-J., Hsueh, P-R., Ko, W-C., Luh, K-T., Tsai, S-H., Wu, H-M. & J-J. Wu, 2001, Metallo-β-latamases in clinical *Pseudomonas* isolates in Taiwan and identification of VIM-3, a novel varient of the VIM-2 enzyme. *Antimicrobial Agents and Chemotherapy*, **45**, 224-228.

Zeng, Q., Stålhandske, C., Anderson, M. C., Scott, R. A. & A. O. Summers, 1998, The core metal-recognition domain of MerR. *Biochemistry*, **37**, 15885-15895.

Zscheck K. K. & B. E. Murray, 1990, Evidence for a staphylococcal-like mercury resistance gene in *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy*, **34**, 1287-1289